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(54) Title: GENE THERAPY FOR REGULATING SMOOTH MUSCLE CELL TONE

(57) Abstract

The present invention is directed towards a method of gene therapy comprising the delivery and expression of a DNA sequence which encodes a protein involved in the regulation of smooth muscle tone in a smooth muscle cell. Also provided by the present invention is a method of regulating smooth muscle tone in a subject comprising the introduction and expression of a DNA sequence encoding a protein involved in the regulation of smooth muscle tone into a sufficient number of cells of the subject to regulate muscle tone in the subject. The present invention also provides a recombinant viral and non–viral vectors comprising DNA encoding a protein involved in the regulation of smooth muscle tone. Also provided by the present invention is a smooth muscle cell which expresses a gene encoding a protein involved in the regulation of smooth muscle tone.

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GENE THERAPY FOR REGULATING SMOOTH MUSCLE CELL TONE

Background of the Invention

There are many physiological dysfunctions which are caused by the deregulation of smooth muscle tone. Among these dysfunctions are erectile dysfuntion and genitourinary dysfunctions of the bladder, ureters, urinary tract, prostate gland, vas deferens, endopelvic fascia and urethra.

Erectile dysfunction is a common illness that is estimated to affect 10 to 30 million men in the United States (Feldman, et al. *Journal of Clinical Epidemiology* 47.5 (1994) 457-67; Anonymous, *International Journal of Impotence Research* 5.4 (1993) 181-284). Among the primary disease-related causes of erectile dysfunction are atherosclerosis, diabetes, aging, hypertension and antihypertensive medication, chronic renal disease, pelvic surgery and radiation therapy, and psychological anxiety (Feldman, et al. *Journal of Clinical Epidemiology* 47.5 (1994) 457-67). Direct cures for the vascular ravages of these manifold and multifaceted disease states are unlikely to occur in the near future, and thus, the last decade has witnessed the development of several treatment modalities to directly restore diminished erectile capacity. However, all currently available therapies are either non-specific (hormonal therapy), of limited overall success (e.g., vacuum erection devices), invasive (e.g., intracorporal injection therapy) or non-reversible and expensive (e.g., penile prosthetic implant surgery). Despite these therapeutic limitations, the FDA approval of CAVERJECT® (July 6, 1995) for intracavernous treatment of erectile dysfunction, of MUSE® (November 19, 1996) for intraurethral drug administration in the treatment of erectile dysfunction, and of Viagra® (March 27, 1998) as an oral therapeutic agent for treatment of erectile dysfunction, represent major steps forward. In essence, these acts of the Federal Government have resulted in both the formal recognition of the medical nature of the problem, and furthermore, legitimized its clinical treatment.

Recent changes in contemporary cultural patterns in the United States have allowed for a free and more open public discussion of sex and sexual dysfunction. This cultural trend has both highlighted the magnitude of the

problem, and simultaneously emphasized the need for improved clinical treatment of erectile dysfunction. The recent deluge of advertising and media activity related to the discussion and treatment of the problem has made men, and their sexual partners, more aware that erectile dysfunction is a common problem with legitimate (federally approved) clinical treatments available. This combination of events will continue to promote even larger numbers of men to seek treatment for impotence from their physicians during the next decade. The magnitude of the problem and the desire for more effective therapies are highlighted by the nearly 2 million prescriptions written for VIAGRA since April of 1998. There is thus now a well-recognized need for a better understanding the impact of age and disease on human erection through the study of the function of the corporal and arterial smooth muscle at the whole tissue, cellular, and most recently at the subcellular level. Also needed is a research strategy that will enable the direct translation of the results of laboratory work to the clinical environment, ensuring that new treatments for organic erectile dysfunction will be more cost effective, of greater efficacy, of longer duration, and with fewer side effects.

Studies have documented that altered corporal smooth muscle tone, resulting in either heightened contractility or impaired relaxation, is a proximal cause of erectile dysfunction in a large proportion of impotent men. These studies have further indicated that complete relaxation of the corporal smooth muscle is both a necessary and sufficient condition to restore erectile potency, unless severe arterial disease or congenital structural abnormalities exist; the latter is true in a minority of patients. The efficacy of recently approved therapies for treating erectile dysfunction that involve agents for directly or indirectly bringing about smooth muscle relaxation - including PGE_1 (CAVERJECT®, EDEX® and MUSE®) and Sildenafil (VIAGRA®) - verifies the validity of this supposition.

As described above, the critical role played by the corporal smooth muscle cells in erectile function makes them an excellent target for molecular intervention in the treatment of smooth muscle dysfunction. Previous efforts have focused on techniques for gene transfer into vascular smooth muscle cells as a basis for the potential therapy of several cardiovascular diseases. Among these are

atherosclerosis, vasculitis and restenosis after balloon angioplasty. These initial studies have provided important information on the efficiency and persistence of gene transfer methods in smooth muscle cells (Finkel, et al. *FASEB Journal* 9 (1995) 843-51).

Thus, because erectile dysfunction is largely caused by altered smooth muscle tone, a method of gene therapy which targets the genes involved in the alteration of smooth muscle tone is extremely desirable. Further, of critical importance with respect to all *in vivo* gene therapy approaches is the percentage of target cells that must be affected, and the relative efficiency of affecting only the desired cell type(s) to see a physiologically relevant therapeutic effect. Accordingly, there is a need for a method of gene therapy wherein only a small number of cells would need to be genetically modified to effect global changes in tissue function. Finally, a successful method of gene therapy for alleviating erectile dysfunction is in great demand as it would be a preferred alternative to currently used methods.

Abnormal bladder function is another common problem which significantly affects the quality of life of millions of men and women. Many common diseases (i.e., stroke, benign prostatic hyperplasia, diabetes mellitus, and multiple sclerosis) alter normal bladder function. Significant untoward changes in bladder function are also a normal occurrence of advancing age. There are two principal clinical manifestations of altered bladder physiology; that is, the bladders may be atonic or hyperreflexic.

The atonic bladder has diminished capacity to empty its urine contents because of an ineffective detrusor smooth muscle (the outer smooth muscle of the bladder wall) contractility. In the atonic state diminished smooth muscle contractility is implicated in the etiology of bladder dysfunction. Thus it is not surprising that pharmacological modulation of smooth muscle tone is insufficient to correct the underlying problem. In fact, the prevailing method of treating that condition is with clean intermittent catheterization. This is a successful means of preventing chronic urinary tract infection, pyelonephritis and eventual renal failure. As such, treatment of the atonic bladder ameliorates the symptoms of

disease, but does not correct the underlying cause.

Conversely, the hyperreflexic, or uninhibited, bladder contracts spontaneously so that the individual may be unable to control the passage of urine with resultant urge incontinence. This is a more difficult problem to treat. Medications that have been used to treat this problem are usually only partially effective and have severe side effects that limit patient use and enthusiasm. The currently accepted treatment options (e.g., oxybutynin, tolteradine) are largely nonspecific, and most frequently involve blockade of the muscarinic receptor and/or the calcium channels on the bladder myocytes. Given the central importance of these two pathways to cellular function in many organ systems throughout the body, such therapeutic strategies are not only crude methods for modulating bladder smooth muscle tone, but, because of their very mechanism(s) of action, are virtually guaranteed to have significant and undesirable systemic effects. Therefore, there is a great need for improved treatment options for bladder dysfunction.

With life expectancy still increasing the incidence of bladder dysfunction will only continue to rise. Based on the extensive evidence already accumulated in another urogenital smooth muscle cell type, namely, the corporal smooth muscle cell, the inventors strongly believe that specific end organ modulation of bladder myocyte tone is the best strategy for correcting bladder dysfunction. There are some physiologically relevant parallels between penile physiology and bladder physiology that bear comparison.

For example, alterations in the tone of the detrusor smooth muscle cell play a similar role in the etiology of bladder dysfunction, to the well-characterized role of the corporal smooth muscle cell in erectile dysfunction. In this regard, the hyperreflexic bladder is characterized by heightened contractility while the atonic bladder is characterized by impaired contractility. Pharmacological therapy for treating bladder hyperreflexia typically involves frequent intravesicular instillations, a treatment that patients often find inconvenient or otherwise undesirable.

In short, frequent intravesicular instillations to restore bladder myocyte function are undesirable and systemic medications still lack tolerable specificity. However, the critical role played by the detrusor smooth muscle cells in bladder function and their accessibility across the urothelium through intravesical instillation makes them a excellent target for molecular intervention in the treatment of bladder dysfunction. Thus, because bladder dysfunction is largely caused by altered smooth muscle tone, a method of gene therapy which targets the genes involved in the alteration of smooth muscle tone is extremely desirable.

Current methods of gene therapy use retroviral or adenoviral based recombinant vectors to infect a target cell. Retroviral and adenoviral based vectors, however, present certain problems which to this date have not been solved. For instance, retroviral and adenoviral based vectors most often will elicit an immune response from the subject being treated. Because of this immune response, the vectors cannot be maintained in the cells, and the DNA is thus not transcribed from the vectors. Furthermore, in order for a global change to occur in the cells surrounding an infected cell, each cell must be infected individually by the recombinant adenoviral vector. Thus, this method of gene therapy is reliant upon the efficiency of transfection. Other side effects from using retroviral or adenoviral based vectors include insertional mutagenesis. Thus, there is clearly a need for a method of gene therapy which does not use retroviral or adenoviral-based vectors, and at the same time is not reliant upon the efficiency of transfection of a vector, but is able to effect global changes in a tissue by modification of only a fraction of the cells.

Summary of the Invention

The present invention is directed towards gene therapy for regulating smooth muscle tone through the delivery of a DNA sequence encoding a protein involved in the regulation of smooth muscle tone into a smooth muscle cell.

The present invention also provides methods of gene therapy wherein the DNA sequence which encodes a protein involved in the regulation of smooth muscle cells encodes a protein which modulates contraction. These proteins will,

for example, enhance smooth muscle contraction thereby restoring smooth muscle tone.

The present invention also provides methods of gene therapy wherein the DNA sequence which encodes a protein involved in the regulation of smooth muscle cells encodes a protein which modulates relaxation. These proteins will enhance smooth muscle relaxation thereby restoring smooth muscle tone.

Further provided by the present invention is a method of inducing smooth muscle cell tone in a subject comprising the introduction and expression of a DNA sequence encoding a protein involved in the regulation of smooth muscle tone into a sufficient number of cells of a subject to alter smooth muscle tone in the subject.

In a specific embodiment of the invention, the methods of gene therapy described herein are used to alleviate bladder dysfunction. In another embodiment of the invention, the methods of gene therapy are used to alleviate erectile dysfunction. In other embodiments of the invention, the methods of gene therapy are used to alleviate dysfunctions of the urethra, prostate, ureter, urinary tract, endopelvic fascia and vas deferens.

The present invention also provides viral and non-viral recombinant vectors comprising a nucleic acid sequence encoding a protein involved in the regulation of smooth muscle tone.

The present invention additionally provides for a smooth muscle cell that expresses a DNA sequence encoding a protein involved in the regulation of smooth muscle tone.

Additional objects of the invention will be apparent from the description which follows.

Brief Description of the Figures

Figure 1 illustrates how selective blockade of the maxi-K channel alters the NTG-induced relaxation response in isolated human corporal tissue strips. A modification of a previously published kinetic protocol was used (Christ, et al., *American Journal of Physiology* 263:H15-H19 (1992)) to derive two parameters; $\tau_{1/2}$: the time elapsed from addition of NTG to achievement of 50% of the steady-

state relaxation response; RSS: the steady-state magnitude of the NTG-induced relaxation response. Note that preincubation of isolated corporal tissue strips resulted in a significant reduction in the magnitude of the NTG-induced relaxation response (100 nM). PE=Phenylephrine; NTG=Nitroglycerine.

Figure 2 sets forth an illustration of K channel function and the control of corporal smooth muscle tone. (+) denotes a positive or stimulatory effect; (-) denoted a negative or inhibitory effect; ? denoted an unknown action; PIP_2: phosphatidylinositol bisphosphate; DAG: diacylglcerol; IP_3: inositoltrisphosphate; NO^-: nitric oxide; NTG: the nitrate donor nitroglycerin; ET-1: endothelin-1; PE: phenylephrine; L-type Ca^{2+}: L-type, voltage dependent calcium channel.

Figure 3 illustrates the surgical preparation and placement of pressure monitoring cannulae.

Figure 4 sets forth the results of experiments determining the fractional change in intracavernous pressure (ICP) in response to neurostimulation. Figure 5 sets forth a diagram indicating the major mechanisms regulating corporal smooth muscle tone. Shown are two corporal smooth muscle cells, interconnected by a gap junction plaque at their lateral border. Also shown are voltage-dependent Ca channels, and K channels. The left cell depicts the series of intracellular events linked to corporal smooth muscle contraction (an elevation in intracellular calcium levels), while the right depicts the series of intracellular events linked to corporal smooth muscle relaxation (a decrease in intracellular Ca^{2+} levels). +: means a stimulatory, positive or increasing effect, and -: means a inhibitory or negative effect

Figure 6 sets forth a photograph of the results of a gene transfer experiment in the rat bladder. The bladder on the left received invesicular distillation of 65 μg of Lac Z cDNA. The bladder on the left is the control bladder.

Figures 7A and 7B set forth the results of a histologic analysis of naked pCMVβ/Lac Z gene transfer into rat corpora *in vivo*. Figure 7A shows intact corpus cavernosum from rat injected with pCMVβ plasmid, containing the Lac Z gene, leading to the expression of β-galactosidase activity and commensurate formation of the chromogenic substrate and characteristic blue color. This photograph was

taken 60 days after the original injection. Note that the robust blue staining is largely confined to the injected corpora, most heavily near the injection site (arrow). Figure 7B shows corpus cavernosum from an age-matched control rat that was not injected with plasmid. Note the complete absence of any blue staining in the corporal tissue (arrow) of this uninjected control animal.

Figure 8 sets forth a representative time course of changes in mean arterial blood pressure (open symbols) and intracorporal pressure (closed symbols) during neurostimulation (2 mA) for an age-matched control, maxi-K transfected and a young control animal. As described in the Examples, the maxi-K transfected animal was subject to a single intracavernous injection of the pcDNA/*hSlo* naked DNA three months prior to this experiment, while the age-matched control animal received the pcDNA vector only in the absence of the *hSlo* cDNA. Note the dramatic differences in the mean amplitude of the intracavernous pressure responses between the maxi-K transfected and age-matched control animals, despite the similarity in mean arterial blood pressure.

Figure 9 shows a summary of all *in vivo* data for intracavernous pressure measurements in response to electrical stimulation of the cavernous nerve in maxi-K transfected, age-matched control and young control animals. Shown are the mean ± S.E.M. for the mean amplitude of the intracavernous pressure responses recorded in all animals in each treatment category, at every level of current stimulation examined in these studies. Note that for the purposes of this illustrative comparison, all of the maxi-K transfected and age-matched control animals were combined as a single population. Where N refers to the total number of animals in each treatment category.

Figures 10A-10C show the mean value of the mean amplitude of the intracavernous pressure responses of the maxi-K transfected animals (>9 months old at the time of injection), 1 month (Figure 10A), 2 months (Figure 10B) and 3-4 months (Figure 10C) after a single intracavernous injection of pcDNA/*hSlo* naked DNA. For the purposes of statistical analysis, all of the age-matched control animals were considered to represent a single homogenous population (see Examples). The numbers (N) in parentheses refer to the total number of

observations for each level of nerve stimulation. The numbers (N) given in the legends above the graphs refer to the total number of animals in that particular treatment group. The number of observations at each level of neurostimulation for the age-matched control animals are shown only in Figure 10A, but were the same for all panels. ICP: intracavernous pressure; BP: mean blood pressure. All data are presented as the mean ± S.E.M. of the mean amplitude of the intracavernous pressure response for each level of current stimulation. 2-way analysis of variance revealed that there were significant differences in the mean amplitude of the intracavernous pressure response at all levels of neurostimulation at the 1 & 2 month time points post-injection. A similar trend was observed even at 3-4 months post-injection; although the small number of observations precluded statistical comparisons.

Figures 11A-11C set forth the results of an RT-PCR assay for gene expression in corporal tissue excised from recombinant human K_{Ca} (*hSlo*) transfected and control rats, 2 months after a single intracorporal microinjection of either pcDNA/K_{Ca} or pcDNA alone (i.e., vector alone). Total RNA was then extracted from pcDNA/K_{Ca}-transfected as well as pcDNA-transfected control tissues, and was RT-PCR amplified with primers as described in the Examples. As shown, the amplification to the 5'-untranslated region resulted in a significant cDNA band from the pcDNA/K_{Ca}-transfected (Lane 4, 5, 6 in Figure 11A), but not from the pcDNA-transfected control tissue (Lane 1, 2, 3 in Figure 11A). The full-length insert with vector 5' and 3' untranslated sequences was also RT-PCR amplified from the pcDNA/K_{Ca}-transfected tissue (Lane 2 in Figure 11C), but not from the pcDNA-transfected control (Lane 2 in Figure 11C), but not from the pcDNA-transfected control (Lane 1 in Figure 11C). The quality of the RNA form both groups of tissue was also examined with the primers that amplified the endogenous K_{Ca}. As indicated in Figure 11B, the endogenous K_{Ca} was amplified to a comparable level in the RNA from all tissues (pcDNA/K_{Ca}-transfected tissue: Lane 4, 5, 6: pcDNA control: Lane 1, 2, 3). The sequences for these primers are described in the Examples. Note that each lane in Figures 11A and 11C correspond to a corporal tissue sample obtained from a distinct animal (n=4).

However, the samples run in lanes 1-6 in Figure 11B were obtained from the same corporal tissues shown in Figure 11A. As such, these data were obtained on a total of 4 distinct *hSlo*-transfected, and 4 distinct vector only rats at the two month time point.

Figure 12 sets forth the results of a Northern blot showing incorporation of a recombinant human K_{Ca} into rat corporal smooth muscle. Total RNA (20 μg each) was obtained from recombinant human K_{Ca} (pcDNA/K_{Ca})-transfected, pcDNA-transfected control, or an uninjected control rat corporal tissue, and was hybridized with biotin-labeled *hSlo* cDNA full length probe for the human sequence. As shown, this full length *hSlo* cDNA detects a band in the pcDNA/K_{Ca}-transfected rat tissue, but not in pcDNA-transfected, nor in the uninjected control rat tissue. The size of this recombinant human K_{Ca} mRNA is ~4.0 Kb. Note that the sequence homology of the rat and human K_{Ca} channels is approximately 90%, which corresponds to about 350 base pair differences. Under the high stringency conditions used in this assay (see Examples), therefore, it would be expected that the full length probe would recognize only the recombinant K_{Ca}, and not the endogenous K_{Ca} sequence.

Figure 13 represents a schematic diagram depicting the proposed model for the "syncytial tissue triad". The concept advanced here is that responses among the vast network of largely inexcitable corporal smooth muscle cells (nominally the final effector of erectile capacity) are primarily coordinated by a triumvirate of mechanisms: 1. Transduction: those intracellular processes that occur following cellular activation (i.e., intracellular signal transduction pathways); 2. Propagation: those processes (i.e., intercellular communication through gap junctions) that permit cells not directly activated by a locally restricted neural or hormonal signal to contribute to the ensuing tissue response (i.e., erection); and 3. Innervation: the characteristics of the effector neural pathways in the penis that are responsible for initiating both erection and detumescence (i.e., innervation density, firing rate, etc.).

Figures 14A and 14B represent voltage dependence of HBSM (human bladder smooth muscle) gap junction channels. Figure 14A shows macroscopic

junctional current (i_j) (Shown on the bottom) during a 2.5-s pulse (10 mV steps to ± 100 mV Shown on top) undergoes a time-dependent decay when transjunctional voltages (V_j) exceeds ± 50 mV. Each voltage pulse was followed by a 5-s recovery interval. Currents were low pass-filtered at a frequency of 1000 Hz and digitized at 4 kHz. The instantaneous (inst) and steady-state (ss) i_j for the experiments shown in Figure 14A were plotted as a function of V_j. The instantaneous i_j - V_j relationships were approximately linear during the ± 100 mV V pulses with the slope conductances 15.4 nS. The steady-state i_j - V_j relationships deviate from linearity above ± 50 mV.

Figure 15 represents steady-state functional conductance-voltage relationships of HBSM (human bladder smooth muscle) cell pairs. Ratios of steady-state (ss) to instantaneous (inst) conductance taken from eleven HBSM cell pairs with voltage protocols identical to those described in the previous Figure. Each point represents a normalized G_j at corresponding V_j. G_j declines symmetrically in both V_j direction and with the greatest decrease in G_j occurring when V_j exceeds ± 50 mV. The solid lines are theoretical fit of the data assuming a two-state Boltzmann distribution (see Examples).

Figures 16A-16C represent single gap junction channel activity of HBSM (human bladder smooth muscle) cell pair: Figure 16A shows whole cell current from a cultured HBSM cell pair during a -30 mV voltage step applied to cell 1 from holding potential of 0 mV. Junctional current appear as equal amplitude and opposite polarity signals (only the junctional current trace was displayed in the Figure). A 70-s segment of 180-s recording is illustrated. Here, three distinct current levels (closed, open current level 1 and level 2) were labeled. The current trace were low pass fileted at 100 Hz and digitized at 1 kHz. Figure 16C shows ll points histogram compiled from the junctional current trade. Three distinct peaks corresponding to three current levels labeled above given the open currents 4.4 and 2.67 pA. The open probability calculated by fitting the histogram was 0.76, and there were a total of 34 channel events and with channel mean open time of 3772 ms. Figure 16C shows composed single channel current-voltage relationship for 8 cell pairs with a slope conductance of 126 pS.

Figure 17A shows whole cell current trace from a bladder cell pair during a +40mV V_j step. Experiment and data analysis procedures are similar to those presented in Figures 16A-16C. There were three distinct conductance levels and a closed state are observed. Figure 17B shows an all point amplitude histogram compiled from the entire 100 sec record (first 10 sec was omitted due to non-stationary). The measured single channel conductance are 80 and 140 pS respectively. Open probability (PO)=0.45; Mean open time (MOT) = 1.41s; Total channel transitions are 65.

Figure 18A shows whole cell current trace from the third bladder cell pair under the identical experiment condition are illustrated here. Three distinct current levels (two fully open channel and one residual state) and one closed state are observed. Figure 18B shows the blow-out of Figure 18A segment indicated by the long arrow to illustrate the transition between the main state and residual state as well as the closed state (indicated by a short arrow). Figure 18C shows an all point histogram compiled from the entire 60s shown on the Figure 18A. Three distinct current peaks give rise to channel conductance's 130 pS, 136 pS for the two fully open channel states, and 36 pS for the residual state.

Detailed Description of the Invention

The present invention provides a method of gene therapy for treating physiological dysfunctions of smooth muscle through the delivery and expression of a DNA sequence encoding a protein involved in the regulation of smooth muscle tone into a smooth muscle cell. As used herein, "regulation" is the modulation of relaxation or the modulation of contraction.

The present invention specifically provides a method of gene therapy wherein the protein involved in the regulation of smooth muscle tone modulates relaxation. Examples of proteins which modulate relaxation include, for example, connexin 43, nitric oxide synthase, guanylate cyclase, adenylate cyclase, protein kinase G, protein kinase A, calcium channels, potassium channels, the K_{ATP} and maxi-K subtypes, and any combination thereof. These proteins will decrease smooth muscle tone and will, for example, result in an increased bladder capacity.

In this embodiment of the invention, the gene therapy method is particularly useful for treating individuals with bladder hyperreflexia. As used herein, a "hyperreflexic bladder" is one which contracts spontaneously so that an individual is unable to control the passage of urine. This urinary disorder is more commonly called urge incontinence, and may include urge incontinence combined with stress incontinence.

Further provided by the present invention is a method of gene therapy wherein the protein involved in the regulation of smooth muscle tone modulates contraction. Examples of proteins which modulate contraction include, for example, connexin 43, alpha 1 receptor or the endothelin 1 receptor, diacylglycerol, protein kinase C, phospholipase C, myosin light chain kinase, calmodulin, potassium channels, calcium channels, and any combination thereof. Specific examples of potassium channels which may be employed are the K_{ATP} and maxi-K subtypes. These proteins can be altered to increase smooth muscle tone, resulting in an increased capacity of a person to empty the urine contents from an atonic bladder. As used herein, an "atonic bladder" is one which has diminished capacity to empty its urine contents because of ineffective detrusor contractility.

It is to be understood that the method of gene therapy described by the present invention may involve the transfer of more than one nucleic acid sequence encoding a protein into a smooth muscle cell whose function is involved in contraction and/or relaxation. For example, the method of gene therapy may involve the transfer of a nucleic acid encoding protein kinase C and the previous, simultaneous or subsequent transfer of a nucleic acid encoding connexin 43. As indicated previously, connexin 43 is a gap junction protein found in bladder and many other smooth muscle cell types which facilitates the interactions between smooth muscle cells. Thus, the addition of nucleic acid encoding connexin 43 will facilitate intercellular interactions so that a greater percentage of cells will be affected by the gene therapy. The presence of this protein in human bladder smooth muscle has been documented only by the inventors.

Also contemplated for use in the method of the present invention is a DNA sequence that encodes a protein that acts to inhibit a protein that modulates

contraction of smooth muscle. An example of a protein that modulates contraction of corporal smooth muscle is protein kinase C. Proteins which inhibit those proteins that are involved in the contraction of the smooth muscle cell will ultimately cause enhanced corporal smooth muscle relaxation resulting in a decreased capacity to empty the bladder of its urine contents. Proteins that inhibit those proteins that are involved in the contraction of the smooth muscle cell will also result in a more easily attained erection.

Further contemplated is a DNA sequence that encodes a protein that acts to inhibit a protein that modulates relaxation of smooth muscle. An example of a protein that modulates relaxation of smooth muscle is protein kinase A. Proteins which inhibit those proteins that are involved in the relaxation of the smooth muscle cell will ultimately cause enhanced smooth muscle contraction resulting in, for example, an increased capacity of a person to empty the contents of their atonic bladder.

Examples of smooth muscle cells for which the method of gene therapy may be used include, but are not limited to, smooth muscle cells of the bladder, urethra (corpus spongiosum), ureter, urinary tract, vas deferens, prostate and penis (corpus cavernosum) as well as the smooth and/or skeletal muscle of the endopelvic fascia. Given the many gross histological and physiological similarities in the factors that regulate tone between smooth muscle cells and other vascular tissue, it follows naturally that similar principles would apply to the arterial smooth muscle cells of the bladder, urethra, ureter, urinary tract, prostate, vas deferens and penis.

The DNA sequence of interest may be introduced into a smooth muscle cell by a number of procedures known to one skilled in the art, such as electroporation, DEAE Dextran, monocationic liposome fusion, polycationic liposome fusion, protoplast fusion, DNA coated microprojectile bombardment, by creation of an *in vivo* electrical field, injection with recombinant replication-defective viruses, homologous recombination, and naked DNA transfer by, for example, intravesicular instillation. It is to be appreciated by one skilled in the art that any of the above methods of DNA transfer may be combined.

In a preferred embodiment of the invention, the DNA for transfer is contained in an aqueous solution. The DNA is preferably transferred into the smooth muscle cells by naked DNA transfer using a mammalian vector. Where the DNA is transferred into smooth muscle cells of the bladder, it is introduced into the bladder by intravesicular instillation, which is a well-established therapy for the treatment of bladder tumors. The DNA solution is then voluntarily withheld by the patient within the bladder for a prescribed duration of time. In another embodiment, the DNA is introduced into the urethra, ureter, upper urinary tract, prostate or endopelvic fascia or vas deferens by instillation or injection therapy and the urethra, upper urinary tract, or ureter is obstructed so that the DNA solution remains in contact with the internal epithelial layer for a prescribed period of time.

The present invention also provides a method of regulating bladder smooth muscle tone in a subject comprising the introduction of a DNA sequence encoding a protein involved in the regulation of smooth muscle tone into a sufficient number of smooth muscle cells of the subject to enhance bladder relaxation in the subject. In a preferred embodiment, the method of the present invention is used to alleviate a hyperreflexic bladder. A hyperreflexic bladder may result from a variety of disorders including neurogenic and arteriogenic, as well as other conditions which cause incomplete relaxation or heightened contractility of the smooth muscle of the bladder. The subject may be animal or human, and is preferably human.

The present invention further provides a method of inducing bladder contraction in a subject comprising the introduction of a DNA sequence encoding a protein involved in the regulation of smooth muscle tone into a sufficient number of smooth muscle cells of the subject to alter bladder contraction in the subject. In a preferred embodiment, the method of the present invention is used to alleviate an atonic bladder. An atonic bladder may result from a variety of disorders including neurogenic and arteriogenic as well as other conditions which cause incomplete contraction of the smooth muscle of the bladder.

A neurogenic bladder dysfunction may manifest itself as partial or complete urinary retention or overflow incontinence. Examples of neurogenic

dysfunctions of the bladder include a hypotonic, or flaccid bladder, and a spastic, or contracted bladder. These dysfunctions may result from an abnormality, injury, or disease process of the brain, spinal cord, or local nerve supply to the bladder and its outlet. Disease processes that result in neurogenic bladder dysfuction include benign prostatic hyperplasia (BPH), syphilis, diabetes mellitus, brain or spinal cord tumors, cerebrovascular accidents, ruptured intervertebral disk, and the demyelinating or degenerative diseases such as multiple sclerosis and amyotrophic lateral sclerosis.

In another embodiment of the invention, the method of the present invention is used to induce contraction of the endopelvic fascia of a subject. The endopelvic fascia contributes to the support of the uterus and vagina. Often times, women who experience childbirth many times will become incontinent due to stress damage between the endopelvic fascia and the muscles of the urethra.

In a further embodiment of the invention, the method of the present invention is used to prevent the hypertrophy of the bladder smooth muscle of a subject caused by benign prostate hyperplasia, by introducing a DNA sequence encoding a protein involved in the regulation of smooth muscle tone into the prostate of the subject. Benign hyperplasia of the prostate gland (BHP) is thought to be associated with increased tone of the smooth muscle cells in the prostate stoma. Eventually BPH results in bladder outlet obstruction and hypertrophy of the bladder.

In addition, the present invention provides for a method of reducing inflammation and irritation of smooth muscle in a subject comprising the introduction and expression of a DNA sequence encoding a protein involved in the regulation of smooth muscle tone into a sufficient number of smooth muscle cells of the subject to reduce the effects of inflammation and irritation. For example, the method provided by the present invention may be used to reduce the symptoms of interstitial cystitis of the bladder. Interstitial cystitis is an example of a condition of the bladder that has clinical manifestations of inflammation and irritation. The interstitial cystitis may be caused, for example, by a collagen disease, an autoimmune disease or an allergic reaction. Furthermore, the method of gene

therapy provided herein may be used, for example, to reduce inflammation and irritation of the urinary tract, ureter or urethra of a subject which may be caused by a bacterial, parasitic or fungal infection through the effects of gene therapy on smooth muscle cells.

Also provided by the present invention is a method of inducing penile erection in a subject comprising the introduction and expression of a DNA sequence encoding a protein involved in the regulation of smooth muscle tone into a sufficient number of smooth muscle cells of the subject to induce penile erection in the subject. In a preferred embodiment, the method of the present invention is used to alleviate erectile dysfunction.

Also contemplated by the present invention is a method of inducing smooth muscle tone in a subject that has undergone reanastomosis of the vas deferens comprising the introduction and expression of a DNA sequence encoding a protein involved in the regulation of smooth muscle tone into a sufficient number of smooth muscle cells of the subject to alter muscle tone in the vas deferens of the subject and ensure propulsion of the contents of the vas deferens.

The introduction of the DNA sequence into the smooth muscle cells of a subject may be effected by methods known to one skilled in the art, such as electroporation, DEAE Dextran, cationic liposome fusion, protoplast fusion, by creation of an *in vivo* electrical field, DNA coated microprojectile bombardment, injection with recombinant replication-defective viruses, homologous recombination, and naked DNA transfer by, for example, intravesicular instillation. It will be appreciated by those skilled in the art that any of the above methods of DNA transfer may be combined. In a preferred embodiment of the invention, the DNA sequence is introduced into the smooth muscle cells by naked DNA transfer.

For the purposes of naked DNA transfer into smooth muscle cells, the DNA sequence may be combined with a sterile aqueous solution which is preferably isotonic with the blood of the recipient. Such formulations may be prepared by suspending the DNA in water containing physiologically compatible substances such as sodium chloride, glycine, and the like, and having buffered pH compatible with physiological conditions to produce an aqueous solution, and rendering such

solution sterile. In a preferred embodiment of the invention, the DNA is combined with a 20-25% sucrose in saline solution in preparation for introduction into a smooth muscle cell.

The DNA sequence for expression may also be incorporated into cationic liposomes and directly injected into the smooth muscle cells of the subject. In a preferred embodiment of the invention, the transfer of the DNA sequence encoding a protein involved in the regulation of smooth muscle tone is performed by the transfer of naked DNA.

The present invention also provides viral and non-viral recombinant vectors. A viral based vector comprises (1) the nucleic acid of or corresponding to at least a portion of the genome of a virus, which portion is capable of directing the expression of a DNA sequence, and (2) a DNA sequence encoding a protein involved in the regulation of smooth muscle tone operably linked to the viral nucleic acid and capable of being expressed as a functional gene product in the target cell. The recombinant viral vectors of the present invention may be derived from a variety of viral nucleic acids known to one skilled in the art, e.g. the genomes of HSV, adenovirus, adeno-associated virus, Semiliki Forest virus, vaccinia virus, and other viruses, including RNA and DNA viruses.

The recombinant vectors of the present invention may also contain a nucleotide sequence encoding suitable regulatory elements so as to effect expression of the vector construct in a suitable host cell. As used herein, "expression" refers to the ability of the vector to transcribe the inserted DNA sequence into mRNA so that synthesis of the protein encoded by the inserted nucleic acid can occur. Those skilled in the art will appreciate that a variety of enhancers and promoters are suitable for use in the constructs of the invention, and that the constructs will contain the necessary start, termination, and control sequences for proper transcription and processing of the DNA sequence encoding a protein involved in the regulation of smooth muscle tone when the recombinant vector construct is introduced into a host cell.

The non-viral vectors provided by the present invention for the expression of the DNA sequence encoding a protein involved in the regulation of smooth

muscle tone in a smooth muscle cell may comprise all or a portion of any of the following vectors known to one skilled in the art: pCMVβ (Invitrogen), pcDNA3 (Invitrogen), pET-3d (Novagen), pProEx-1 (Life Technologies), pFastBac 1 (Life Technologies), pSFV (Life Technologies), pcDNA2 (Invitrogen), pSL301 (Invitrogen), pSE280 (Invitrogen), pSE380 (Invitrogen), pSE420 (Invitrogen), pTrcHis A,B,C (Invitrogen), pRSET A,B,C (Invitrogen), pYES2 (Invitrogen), pAC360 (Invitrogen), pVL1392 and pVl1392 (Invitrogen), pCDM8 (Invitrogen), pcDNA I (Invitrogen), pcDNA I(amp) (Invitrogen), pZeoSV (Invitrogen), pRc/CMV (Invitrogen), pRc/RSV (Invitrogen), pREP4 (Invitrogen), pREP7 (Invitrogen), pREP8 (Invitrogen), pREP9 (Invitrogen), pREP10 (Invitrogen), pCEP4 (Invitrogen), pEBVHis (Invitrogen), and λPop6. Other vectors would be apparent to one skilled in the art.

Suitable promoters include, but are not limited to, constitutive promoters, tissue specific promoters, and inducible promoters. In one embodiment of the invention, expression of the DNA sequence encoding a protein involved in the regulation of smooth muscle tone is controlled and affected by the particular vector into which the DNA sequence has been introduced. Some eukaryotic vectors have been engineered so that they are capable of expressing inserted nucleic acids to high levels within the host cell. Such vectors utilize one of a number of powerful promoters to direct the high level of expression. Eukaryotic vectors use promoter-enhancer sequences of viral genes, especially those of tumor viruses. This particular embodiment of the invention provides for regulation of expression of the DNA sequence encoding the protein using inducible promoters. Non-limiting examples of inducible promoters include, but are not limited to, metallothionine promoters and mouse mammary tumor virus promoters. Depending on the vector, expression of the DNA sequence in the smooth muscle cell would be induced by the addition of a specific compound at a certain point in the growth cycle of the cell. Other examples of promoters and enhancers effective for use in the recombinant vectors of the present invention include, but are not limited to, CMV (cytomegalovirus), SV40 (simian virus 40), HSV (herpes simplex virus), EBV (epstein-barr virus), retroviral, adenoviral promoters and enhancers,

and smooth muscle specific promoters and enhancers. An example of a smooth muscle cell specific promoter is SM22α.

The present invention further provides a smooth muscle cell which expresses an exogenous DNA sequence encoding a protein involved in the regulation of smooth muscle tone. As used herein, "exogenous" means any DNA that is introduced into an organism or cell. The introduction of the recombinant vector containing the DNA sequence into the smooth muscle cell may be effected by methods known to one skilled in the art, such as electroporation, DEAE Dextran, cationic liposome fusion, protoplast fusion, DNA coated microprojectile bombardment, injection with recombinant replication-defective viruses, homologous recombination, and naked DNA transfer by, for example, intravesicular instillation. "Naked DNA" is herein defined as DNA contained in a non-viral vector. It will be appreciated by those skilled in the art that any of the above methods of DNA transfer may be combined.

Of critical importance with respect to all *in vivo* gene therapy approaches is the percentage of target cells that must be affected, and the relative efficiency of affecting only the desired cell type(s) to see a physiologically relevant therapeutic effect. The method of gene therapy provided by the present invention meets the need for a method which produces global changes in a tissue by genetically modifying only a fraction of the cells. The inventors have demonstrated the effectiveness of naked DNA transfer in the penis and in the bladder, and have shown that the physiological functions of a smooth muscle cell enable the intercellular transfer and continuous propagation of the effects of incorporation of naked DNA sequences encoding proteins involved in the regulation of smooth muscle tone in only a fraction of the total cellular population. It is a well documented fact that corporal and arterial smooth muscle cells are interconnected by a ubiquitously distributed population of intercellular channels known as gap junction proteins both *in vivo* and *in vitro* (Christ, G.J., et al. *Life Sciences* 49.24 (1991) PL195-200; Christ, G.J., et al. *International Journal of Impotence Research* 5.2 (1993) 77-96; Christ, G.J., et al. *Journal of Pharmacology & Experimental Therapeutics* 266.2 (1993) 1054-65; Christ, G.J., et al. *Biophysical Journal* 67.3

(1994) 1335-44; Christ, G.J., *Urological Clinic of North America*, 22.4 (1995) 727-745; Christ, G.J. *World Journal of Urology* (1997) 15:36-44; Christ, et al., *Circulation Research*, 79 (1996) 631-646; Christ & Melman, *Molecular Urology* (1997) 1:45-54), the mechanisms for which are depicted in Figure 2.

In the corpora, smooth muscle contraction (i.e. elevation in intracellular calcium levels) might be accomplished following activation of the α_1-adrenergic receptor by norepinephrine, or by endothelin-1 activation of the ET_A receptor. In both cases, receptor activation leads to Ca^{2+} mobilization. Specifically, activation of these receptors by norepinephrine or ET-1 leads to activation of phospholipase C, which cleaves membrane bound phosphatidyl inositol (PIP_2) into IP_3 and diacylglycerol (DAG). As illustrated in Figure 1, increases in DAG and IP3 ultimately exert their effects, at least in part, via increase in intracellular Ca^{2+} levels. Conversely, any physiological event resulting in a diminution of transmembrane Ca^{2+} flux, or sequestration of intracellular Ca^{2+}, e.g., membrane hyperpolarization, will result in smooth muscle relaxation. For example, PGE_1 activates the PGE_1 receptor, to stimulate the adenylate cyclase enzyme, which then catalyzes the conversion of ATP to cAMP. Increased cAMP then stimulates protein kinase A (PKA). Alternately, smooth muscle relaxation can be achieved by nitric oxide, released from endothelial or neuronal sources. Nitric oxide diffuses into smooth muscle cells to activate soluble guanylate cyclase, that catalyzes the conversion of GTP to cGMP. Elevated cGMP levels activate protein kinase G (PKG). The effects of PKA, PKG, and PKC on gap junctions, K channels, and Ca^{2+} channels are thought to be mediated via phosphorylation of specific amino acid residues on target proteins (gap junctions, K channels, and Ca^{2+} channels). Figure 2 illustrates the result of their putative actions as follows: +: means a stimulatory, positive or increasing effect, and -: means a inhibitory or negative effect. This simplified model illustrates how the interactions of these important second messenger systems might impact on gap junctions, K^+, and Ca^{2+} channels, and thus modulate smooth muscle tone *in vitro* and *in vivo*. K^+ ion flux is controlled by three main effector pathways, the first two are the cAMP/PKA (PKA: protein kinase A) and cGMP/PKG (protein kinase G) pathways which are activated by PGE_1 and

NO, respectively, and these pathways clearly modulate the activity of the maxi-K channel; their effects on the K_{ATP} channel have not been documented. The third pathway is the potassium channel modulators, which modulate the activity of the K_{ATP} channel. Because of the disposition of these ions in the intra- and extracellular spaces, the opening of Ca^{2+} channels leads to the influx of Ca^{2+} down its electrochemical gradient and subsequent intracellular depolarization, while the opening of K channels leads to efflux of K+ from the cell down its electrochemical gradient, and subsequent intracellular hyperpolarization. The effects of these reciprocal pathways on membrane potential and the level of corporal smooth muscle tone are exerted, at least in part, through modulation of the intracellular calcium concentration, with increases in intracellular calcium associated with contraction, and decreases in intracellular calcium associated with relaxation.

Connexin 43 is the predominant gap junction protein isoform expressed in the human and rat bladder. As in other tissues, these intercellular channels provide partial cytoplasmic continuity between adjacent smooth muscle cells, allowing the intercellular exchange of physiologically relevant ions (K^+ and Ca^{2+}) and second messenger molecules (IP_3, cAMP, cGMP).

While bladder myocytes are clearly capable of regenerative electrical events, the presence of gap junctions is still relevant not only to provide a "safety factor" for the maintenance and propagation of regenerative electrical events (as shown in the heart), but also for ensuring tissue plasticity. Viewed in terms of the recently advanced "syncytial tissue triad" concept (see Christ, G.J., World J Urol (1997) 15:36-44, herein incorporated by reference), the presence of gap junctions is thought to endow the bladder musculature with the ability to function syncytially under a very wide range of physiological conditions. This is an extremely important point given that in the bladder, as in the penis, not every smooth muscle cell is directly innervated by the autonomic nervous system. In fact, the electrical excitability of the bladder myocyte has been traditionally viewed as *a priori* evidence for the lack of importance of demonstrable gap junctions between bladder myocytes (using light and electron microscopy).

Thus, the presence of gap junctions, as demonstrated by both molecular biological and electrophysiological techniques provides an additional anatomic substrate for the rapid and syncytial contraction and relaxation responses required of the bladder smooth muscle cells for normal bladder function. But perhaps more important, these gap junctions may provide an important mechanistic basis for the potential efficacy of gene therapy in the treatment of bladder dysfunction. That is, the presence of gap junctions will permit smooth muscle cells not directly activated by a relevant neuronal/pharmacologic signal to be rapidly, albeit indirectly, recruited into the contraction or relaxation response by this prominent intercellular pathway. The experimental and clinical evidence verifying the validity of this supposition has been outlined in recent publications (Christ, G.J., et al., *Circulation Research*, 79:631-646 (1996); Christ, G.J., *World Journal of Urology*, (1997) 15:36-44), and a mathematical model has been constructed that accounts for this behavior (Christ, G.J., et al., *Journal of Urology* 155:620A (Abstract) (1996); Christ, G.J., et al., *Journal of Theoretical Biology* (1998, in press)). The major implication is that the presence of these gap junction channels makes it likely that only a small fraction of the smooth muscle cells would need to be genetically modified to affect rather global changes in smooth muscle tone. Thus, as the inventors have already demonstrated for the penis, the major implication is that the presence of these gap junction channels insures that only a small fraction of smooth muscle cells would need to be genetically modified to affect rather global changes in bladder and penile smooth muscle tone.

The anatomical location of the human bladder makes gene therapy an attractive possibility because, as with the human penis, the bladder is a relatively "isolated" organ system that should be quite amenable to genetic modification in the absence of effects on other organs/tissues. The bladder is an internal organ into which external substances can be easily introduced through a urethral catheter. BCG therapy for bladder cancer is already a well-recognized use of this technique. The instillation of a gene(s) product, in an aqueous solution that can be voluntarily retained by the patient within the bladder for a prescribed duration of time would be expected to be largely restricted to the bladder musculature. The

data described in the Examples below document that this is indeed the case. In this scenario, there is clearly little risk of systemic side effects.

Further, the method of gene therapy provided by the present invention is designed to take advantage of the generally accepted physiological principle that relatively subtle alterations in the balance between contracting and relaxing stimuli can result in profound alterations in smooth muscle physiology, and thus, smooth muscle function (Lerner, et al. *J. Urol.* 149.5 Pt 2 (1993) 1246-55; Azadzoi, et al. *J. Urol.* 148.5 (1992) 1587-91; Christ, et al. *British Journal of Pharmacology* 101.2 (1990) 375-81; Christ, G.J., *Urological Clinics of North America* 22.4 (1995) 727-745; Taub, et al. *J. Urol.* 42 (1993) 698). The goal of gene therapy is to restore a more normal balance between contracting and relaxing stimuli following expression of exogenous genes that code for physiologically relevant proteins in bladder smooth muscle. As indicated herein in the rat model, expression of these exogenous genes can be maintained for a period of weeks to months. Thus, it would then permit the patient to obtain "normal" bladder function in the absence of any other exogenous manipulation during this time period. Clearly this is major advance over all currently available therapies. Since the inventors have already demonstrated that the gap junction protein Connexin 43, as well as two prominent K^+ channel subtypes (i.e., the maxi-K and K_{ATP} channels) are present in rat and human bladder smooth muscle, these are the first logical targets for evaluating the efficacy of genetic modulation of bladder smooth muscle tone.

Effect of Gene Therapy on Organ Systems

Smooth muscle cells constitute a heterogenous collection of effector cells. By virtue of their presence in, and regulation of, blood vessels they ultimately affect the function of all organs. Moreover, they are the parenchymal cells in most hollow organs, such as those found in the urogenital tract (i.e., bladder, ureter, prostate). Everywhere, including the entire genitourinary system, coordination of activity among the smooth muscle cells is an important component of normal tissue function and, therefore, organ function. While the autonomic nervous

system supplies neural input to all peripheral organs, the density, distribution and roles of nerve fibers vary. In a given tissue the level of activity of smooth cells and the cellular mechanisms involved can differ dramatically among the different smooth muscle phenotypes. Moreover, it is increasingly clear the role of the autonomic nervous system in particular organs is uniquely correlated with the physiological phenotype of the constituent smooth muscle cells. Membrane excitability, signal transduction processes, and the extent of cell-to-cell communication between smooth muscle cells all vary between different tissues. Thus, the components of an effector process are integrated together to yield the characteristic action of an organ. Moreover, the components occur in different combinations to provide a rich diversity of organ function. Understanding the principles of initiation and spread of stimuli in the smooth muscle tissues is clearly necessary for understanding organ physiology, and moreover, as discussed below provides the key to the success of gene therapy.

It is of particular relevance that the functional diversity of smooth muscle correlates with the functional diversity of peripheral organ systems. This diversity is expressed at every level of tissue organization within an organ, from the pattern of the autonomic neuronal innervation to the phenotype of the parenchyma cell. Much of the diversity in smooth muscle function is due to the effector systems that transduce the results of receptor activation into intracellular second messenger events throughout the tissue. Clearly such diversity of smooth muscle regulation/function has important implications for smooth muscle-specific therapeutic options. However, one also needs to consider the mechanism(s) by which signals are integrated among the parenchyma cells. In particular, the success of gene therapy, as addressed in this application, is dependent on the more recent evidence documenting the distribution and function of gap junctions between smooth muscle cells in organ systems throughout the body. This evidence, as briefly reviewed below, points to a major role for intercellular communication in coordination smooth muscle responses in the various diverse tissues in different organs.

The principles outlined above have one major implication for gene therapy in the urogenital system which is counterintuitive to the dogma that governs gene therapy approaches for many other diseases (i.e., cancer, cardiovascular, etc). More specifically, the genetic therapy of such diseases is explicitly dependent on high efficiency transfection rates in selected cells following systemic administration. Such requirements pose significant barriers to clinical success. As such, the treatment of cancer and/or systemic vascular disease stands in stark contrast to the treatment of impotence and incontinence, where the isolated, easily accessible condition of the penis and bladder confers distinct advantages to the selective administration of genetic material. This fact, in conjunction with the documented presence of gap junctions among smooth muscle cells throughout the urogenital tract, and the fact that subtle alterations in the tone of these cells are responsible for many aspects of human urogenital disease provides a clear advantage to gene therapy for these diseases. That is, in the urogenital system, the goal of gene therapy as proposed herein is to achieve the lowest transfection rate possible that is correlated with the desired physiological changes. Taken together, it is clear that less aggressive and invasive genetic strategies, such as a single injection of naked DNA, are attractive options for the effective treatment of urogenital disease. The inventors' physiological studies in the rat penis (Rehman et al., 1997; Christ et al., 1998) confirm this supposition, and thus, have assisted in establishing the boundary conditions for the maintenance of normal integrative tissue responses. Moreover, the parameters/conditions for normal tissue behavior that the inventors have identified thus far have been recently encapsulated into a mathematical construct (Ramanan et al., *J. Theor. Biol.* (1998, in press)). The resulting mathematical model can be used to generate experimentally and clinically testable hypotheses concerning the predicted behaviors of tissues under a diverse range of physiologically relevant conditions. As such, the model will assist, for example, in determining the lower limit of transfection efficiency required to achieve restoration of normal tissue function in a urogenital organ compromised by disease. It will also assist in identifying the best probes/genes for the said transfection.

The inventors anticipate that gene therapy alone, that is, the proposed genetic alteration in how the smooth muscle cells respond to their environment, will work in all patients in whom there is enough neuronal innervation remaining to coordinate a normal syncytial tissue response. However, in those patients in whom sufficient neuronal innervation is not available to guarantee the success of gene therapy per se, the inventors have proposed a secondary strategy. The secondary strategy involves the concomitant administration of either orally active agents, or locally administered (i.e., the MUSE intraurethral suppository) drugs that will augment the underlying effects of gene therapy (in particular penile erection or bladder function). For example, one could transfect the penile or bladder smooth muscle cells with a potassium channel as proposed herein, and then deliver to the patient a drug which selectively activated that channel. There are many possible combinations of custom designed drugs and/or genetically engineered potassium channels that could produce a similarly desired effect. Another example of such a secondary, or combined genetic/pharmacologic therapy would be the transfection of the penis or bladder with a maxi-K or K_{ATP} channel subtype/isoform/chimera, and then giving the patient a selective/synthetic activator of that channel. It is important to point out that these two examples represent merely the most obvious of a plethora of potential therapeutic possibilities.

Figures 13-18 document the presence of the requisite cellular components of human bladder smooth muscle cells that make this organ attractive for genetic therapy (either primary or secondary (i.e., in association with subsequent selective pharmacological manipulation)). As illustrated in the Figures, the gap junction protein connexin 43 is an integral component of the human bladder smooth muscle. To the best of the inventors' knowledge, this is the first demonstration that gap junctions are present in human bladder smooth muscle. They have also recently confirmed the presence of the maxi-K channel in human bladder (data not shown). These two observations, in conjunction with other established properties of the human bladder, make it amenable to the same gene therapy protocols proposed for the human penis, as enumerated throughout this application.

The present invention is described in the following Experimental Details Sections which is set forth to aid in the understanding of the invention, and should not be construed to limit in any way the invention as defined in the claims which follow thereafter.

Experimental Details Section

I. Materials and Methods

A. Erectile Dysfunction Gene Therapy Experiments Using a Recombinant Vector

Plasmids and genes. The pCMVβ and pcDNA3 plasmids were purchased from Invitrogen (San Diego, CA). The human maxi-K cDNA (hSlo) was obtained from Dr. Salkoff (Washington University School of Medicine, St. Louis, MO)(McCobb, D. P., et al. *American Journal of Physiology* 269 (1995) H767-H777. The nucleotide sequence of the hSlo cDNA is also available at Genbank Accession No. U23767. The neuronal NOS cDNA was obtained from Dr. S. Snyder (Johns Hopkins University) and Dr. D. Bredt (Univ. Calif. San Francisco)(Bredt, et al., Nature 351 (1991) 714-8). The nucleotide sequence of the neuronal NOS is also available at Genbank Accession No. X59949. The human maxi-K channel cDNA (approximately 3.9 kb)(McCobb, et al. American Journal of Physiology) 269 (1995) H767-H777) or the neuronal NOS cDNA (Bredt/Snyder 91287795) (Bredt, et al. *Nature* 351 (1991) 714-8) was inserted into the XhoI-XbaI cloning sites of the pcDNA3 vector, where expression is driven off of the CMV promoter (Invitrogen). One hundred micrograms of plasmid DNA was suspended in 200 μl sterile PBS containing 20% sucrose and injected into the corpus cavernosum (CC) of anesthetized rats.

Gene transfer of Lac Z into the corpus cavernosa using various techniques.

Naked DNA. One hundred μg of pCMVβ plasmid (Clonetech, CA) (contains the LacZ gene under the control of the CMV promoter) in 200μl phosphate buffered saline (containing 20% sucrose) were injected into the corpus cavernosum of 3 month old Fischer rats (under anesthesia). Ten days later CC tissue was resected and stained for β-galactosidase activity.

Liposomes. Five micrograms of pCMVβ plasmid in 100μl PBS were mixed with 100 μl Lipofectin reagent (Gibco), and the resultant liposome complexes (200 μl) were injected into corpus cavernosum into 3 month old Fischer rats (under anesthesia). Ten days later CC tissue was resected and stained for β-galactosidase activity.

Adenovirus Vector. Two hundred microliters of Adenovirus vectors ($>10^{10}$ pfu/ml) containing the lacZ cDNA were obtained from Dr. Roy Chowdhury (AECOM gene therapy core) and injected intra CC in 3 month old Fischer rats (under anesthesia). Three days later CC tissue was resected and stained for β-galactosidase activity.

Staining for β-galactosidase activity. CC tissue was excised from rats at various times after injection, fixed with 4% paraformaldehyde/0.1% glutaraldehyde for 3 hours, and stained with X-Gal for 15 hrs. at 37°C (Vitadello, M., et al. *Human Gene Therapy* 5 (1994) 11-8).

Animal. 62 male Sprague Dawley (Taconic Farms, Germantown, NY) 10-20 weeks old, and weighing 200-250g were used in these experiments. All rats were fed Purina lab rodent chow ad *libitum* and housed individually with 07.00-19.00 light cycle. Rats were divided into groups as indicated in Table 1.

Table 1: Gene Transfer and Time Course Experiments

Group	Rats	Age	Gene Therapy	Method of Gene Transfer	Time Course Experiments
A	n=12	10 week	Lac Z (n=9) Control (n=3)	Adenovirus (n=3) Liposome (n=3) Naked DNA (n=3)	
B	n=24	10 week	Lac Z (n=12) Control (n=12)	Naked DNA (Sham)	2 week (n=3) 4 week (n=3) 8 week (n=3) 12 week (n=3) 2 week (n=3) 4 week (n=3) 8 week (n=3) 12 week (n=3)
C	n=20	20 week	NOS (n=12) Control (n=8)	Naked DNA (Sham control)	1 month (n=2) 2 month (n=4) 3 month (n=4) 4 month (n=2) 1 month (n=2) 2 month (n=2) 3 month (n=2) 4 month (n=2)
D	n=18	20 week	Maxi-K (n=10) Control (n=8)	Naked DNA (Sham control)	1 month (n=2) 2 month (n=4) 3 month (n=2) 4 month (n=2) 1 month (n=2) 2 month (n=2) 3 month (n=2) 4 month (n=2)

Preparation of animals for *In vivo* erectile studies.

Induction of anesthesia. The rats were anaesthetized by intraperitoneal injection (35 mg/kg) of sodium pentobarbital (Anpro Pharmaceuticals). Anesthesia was maintained during the course of the experimental protocol (2-3 hrs) by subsequent injection of pentobarbital (5-10 mg/kg) every 45-60 minutes, as required for maintenance of anesthesia.

Surgical preparation and placement of pressure monitoring cannulae. Figure 3 illustrates the entire experimental procedure. Animals were anaesthetized, placed in the supine position, and the bladder and prostate were

exposed through a midline abdominal incision. The arterial line in the left carotid artery was connected to a MacLab data acquisition board via a transducer and transducer amplifier, for continuous monitoring of blood pressure. A right external jugular venous line was utilized for intravenous fluid transfusion or blood sampling. The prostate was exposed by a lower midline incision. The cavernous nerves were seen on the posterolateral surface of the prostate arising from the pelvic ganglion which is formed by the joining of the hypogastric and pelvic nerves. The two corpora were exposed by inguinoscrotal incisions on both sides, combined with degloving of the penis. A line was inserted into the right corpora for continuous monitoring of intracorporal pressure via the MacLab instrumentation. Yet another line was inserted in the left corpora for intracavernous drug injection. Lastly, the nerve stimulator probe was placed around the cavernous for current stimulation. The inferior hypogastric plexus (i.e., the pelvic plexus or major pelvic ganglia), pelvic nerves and the cavernous nerve were identified posterolateral to the prostate on both sides, and the stainless-steel bipolar wire electrodes were placed around these structures for electrical stimulation. Both crura of the penis were exposed by removing part of the overlying ischiocavernous muscle. In order to monitor intracavernous pressure (ICP), a 23-gauge cannula was filled with 250 U/ml of heparin solution, connected to PE-50 tubing (Intramedic, Becton Dickinson) and inserted into the right corpus cavernosum. The tubing was then fixed to the tunica with a 7-0 Dermalon suture, to ensure stability during measurement of ICP. Another 23 gauge cannula was connected to a 1 ml syringe and inserted into left corpus cavernosum for intracavernous drug injection. Systemic arterial blood pressure (BP) was monitored via a 25-gauge cannula placed into the carotid artery.

Both pressure lines (BP) and (ICP) were connected to a pressure transducer, which was, in turn, connected via a Transducer amplifier (ETH 400 CB Sciences, Inc) to a data acquisition board (Mac Lab/ 8e7, ADI Instruments, MA). Real-time display and recording of pressure measurements was performed on a Macintosh computer (Mac Lab software V3.4). The pressure transducers and AID board were calibrated in cm of H_2O.

Neurostimulation of cavernous nerve and recording of intracavernosal pressure. Direct electrostimulation of the cavernous nerve was performed with a delicate stainless-steel bipolar hook electrode attached to the multijointed clamp. Each probe was 0.2 mM in diameter; the two poles were separated by 1 mM. Monophasic rectangular pulses were delivered by a signal generator (custom made and with built in constant current amplifier). Stimulation parameters were as follows: frequency; 20 Hz, pulse width; 0.22 msec, duration; l minute. The current protocol was the application of increasing current at the following intervals: 0.5, l, 2, 4, 6, 8 and 10 mA. The changes in intracavernous pressure and systemic blood pressure were recorded at each level of neurostimulation.

Tissue Procurement and fixation and immunohistochemistry analysis.

Tissue retrieval. Following completion of the neurostimulation experiments the penises of both the gene therapy and age-matched control animals were harvested, and the distal end of the penis marked with methylene blue to ensure the proper later identification of the distal and proximal ends. All penile tissue was immediately transferred to 4% paraformaldehyde in phosphate buffer pH 7.4 for fixation for 4 hours at 20°C, and later cryoprotected in cold 30% sucrose in 0.1M phosphate buffer (PBS; at 4°C overnight or longer), pH 7.4 for immunostaining. Part of the penis of the animal was frozen in liquid nitrogen and preserved at 80°C for molecular biological studies. Briefly, the tissue was sectioned on a cryostat at l4 μM and the sections were dried onto slides and paraffin embedded. The slides were stored at -20 C° until they were stained, which was usually within 2-4 weeks.

Histology. Histological examination of the sections were performed to confirm the identification of nerves and smooth muscles. Serial, slide-mounted sections were fixed in 10% formalin and stained with hematoxylin and eosin. All slide preparation specimens were viewed with Zeiss microscope.

Nitric Oxide Synthase Immunohistochemistry. Slide mounted tissue sections were deparaffinized with xylene, rehydrated in graded alcohols and blocked for endogenous peroxidase activity with 3% hydrogen peroxide.

Nonspecific binding of antibodies to the specimens was blocked by incubation with 1.5% normal goat serum in phosphate-buffered saline (PBS) for 30 minutes at room temperature. The slides were then drained and incubated for one hour at room temperature with primary antibody. The antibody used was a rabbit polyclonal antibody directed against brain NOS (Transduction Laboratories, Lexington Kentucky). An antibody concentration of 1.0 μg/ml was found to be optimal for immunostaining. Antigen binding was detected by the avidin-biotin immunoperoxidase method, using the VectaStain Elite ABC kit. The color reaction was developed with diaminobenzidine activated with hydrogen peroxide (diaminobenzidine as the chromogen) and then counterstained with hematoxylin solution. Staining was not evident in the negative control which substituted PBS for primary antibody, supporting the specificity of primary antibody used in the experiment.

Statistical analysis. All statistical analyses were performed using the Stat-View 4.5 software (Abacus Concepts, Berkeley, CA). A two-tailed Student t test for unrelated samples was utilized for comparison of group means for parameters of interest between gene therapy rats (NOS or maxi-K at different time points post-transfection) and age-matched control and young animals were used as appropriate. All differences were considered significant at $p<O.O5$. Unless otherwise stated, all data are expressed as the mean S.E.M.

Analysis of Neurostimulation Data. Stimulus response curves were generated for neurostimulation by plotting the fractional change in corporal pressure as a function of mean systemic blood pressure (expressed as ICP/BP) for stepwise increases in current (1,2,4,6,8,10 mA)(Sigma Plot Mac V5.0 Jandel Scientific, San Rafael, CA) both for control and gene therapy rats.

Gene transfer of Lac Z into smooth muscle cells of the rat corpus cavernosum. Gene transfer into vascular smooth muscle cells has been achieved using various techniques, such as retroviruses, adenoviruses, cationic liposomes, or naked DNA transfer. To determine the efficiency of these techniques for *in vivo*, intracorpus cavernosal gene transfer, the plasmid pCMβ was injected as naked DNA or incorporated into cationic liposomes, or recombinant adenovirus

containing the Lac Z cDNA (coding for β-galactosidase), each into CC tissue of three rats. All three techniques of gene transfer produced positive results, but adenoviral mediated gene transfer was the most effective, as evidenced, on the whole tissue level, by the conversion of the chromogenic substrate, X-Gal (5-bromo-4-chloro-3-indolyl-b-D-galactoside) into its blue breakdown product by the activity of β-galactosidase.

To determine the relative number and histologic type of cells expressing β-galactosidase, one hundred μg of pCMVβ plasmid in 200μl phosphate buffered saline (containing 20% sucrose) were injected into the CC of 3 month old Fischer rats (under anesthesia). Thirty days later, CC from injected and control rats were excised, fixed with 4% paraformaldehyde/0.1% glutaraldehyde for 3 hours, and reacted with X-Gal for 15 hrs. at 37°C, paraffin embedded and sectioned (Vitadello, M., et al. *Human Gene Therapy* 5 (1994) 11-8). β-galactosidase activity was evident in a significant number of the smooth muscle cells thirty days after DNA injection.

Increased intracorporal pressure following injection of cDNA for neuronal NOS or potassium channel Maxi-K. Either the human maxi-K channel cDNA or the neuronal NOS cDNA was inserted into the XhoI-XbaI cloning sites of the pcDNA3 vector. One hundred μg of each plasmid, in 200μl phosphate buffered saline, (containing 20% sucrose) was injected into the CC of 4 month old Fischer rats (under anesthesia). Control rats were either sham operated, sham operated with an intracorporal injection of 200μl PBS containing 20% sucrose, or sham operated with an intracorporal injection of 200μl PBS containing 20% sucrose and 100 μg pcDNA vector DNA. Basal and nerve-stimulated intracorporal pressures (ICP) were measured between 2 weeks and 4 months after injection. No significant differences were observed in intracorporal pressures within the time frame examined, and results from all animals within each group were pooled. Likewise, no significant differences were observed among the various controls, and all control data was pooled.

The data depicted in Figure 4 and Table 2 indicate that the injection of either the NOS or maxi-K cDNAs significantly increased both basal and

nerve-stimulated ICP. Mean basal fractional change in ICP increased from a control level of ≈8 (cm of H_2O) to ≈ 14 in NOS-injected rats, to ≈13 in maxi-K-injected rats. In a similar manner, the nerve-stimulated fractional change in ICP was approximately 30% higher in both NOS and maxi-K-injected rats, over a range of 2-10 milli amp stimulation.

The age matched control animals were considered to represent a homogenous population, since statistical analysis revealed that there was no significant difference between these animals 1-4 months after receipt, with respect to the mean amplitude of the intracavernous pressures measured in response to all levels of current stimulation used in these studies. In addition, it should be noted that four retired breeders received an injection with the pcDNA/hSlo DNA (group 3), as described below, and then 2 months after this injection, the animals were sacrificed and the corporal tissue quickly excised and flash frozen in liquid nitrogen for RT-PCR and Northern analyses (without the performance of any physiological experiments *in vivo*); again, 5 age-matched control animals were run in parallel, and received injection of vehicle only.

Microinjection of vectors/plasmids into rat corporal tissue. Animals were anesthetized by intraperitoneal injection of sodium pentobarbitol (35 mg/kg). An incision was made through the perineum, the corpus spongiosum was identified, and a window was made in the corpus spongiosum for identification of the corpus cavernosum. All microinjections consisted of a single bolus injection into the corporal tissue, made using an insulin syringe. The final volume of all microinjections was 200 μl.

Gene transfer of LacZ into smooth muscle cells of the rat corpus cavernosum *in vivo*. Transfer of Lac Z DNA (coding for β-galactosidase) into rat corporal smooth muscle cells *in vivo* was accomplished by injecting the plasmid pCMVβ as naked DNA into the corporal tissue of 10 week old sprague Dawley rats, and the corporal tissue was obtained from groups of three animals at each of four time points ranging from 2-11 weeks post injection (see Table 4 for details). An equivalent number of uninjected control animals at each time point post-injection were run in parallel. For these studies, 100 μg of pCMVβ plasmid dissolved in 200

μl phosphate buffered saline (containing 20% sucrose) was injected into the corpus cavernosum of 10 week old Sprague Dawley rats (under anesthesia). 2-11 weeks later, the corporal tissue from injected and control rats were excised, fixed with 4% paraformaldehyde, 0.1% glutaraldehyde for 3 hours, and reacted with X-Gal for 15 hrs. at 37°C, paraffin embedded and sectioned. The efficacy of gene transfer was assessed, on the whole tissue level, by the conversion of the chromogenic substrate, X-Gal (5-bromo-4-chloro-3-indolyl-b-D-galactoside) into its blue breakdown product by the activity of β-galactosidase.

Preparation of plasmids, and transfection of rat corporal smooth muscle *in vivo*. The human maxi-K channel cDNA (*hSlo*) (approximately 3900 nucleotides, i.e., 3.9kb) was inserted into the XhoI-XbaI cloning site of the pcDN3 vector. One hundred μg of pcDNA/maxi-K, in 200 μl phosphate buffered saline, (containing 20% sucrose) was injected into the corpus cavernosum of anesthetized 9 month old sprague Dawley rats. Control rats were either sham operated, sham operated with an intracorporal injection of 200 μl PBS containing 20% sucrose, or sham operated with an intracorporal injection of 200 μl PBS containing 20% sucrose and 100 μg pcDNA vector DNA. Basal and nerve-stimulated intracorporal pressures (ICP) were measured between 1-4 months after the intracavernous injection.

Surgical preparation and placement of pressure monitoring cannulae. Details of this methodology have been recently described (Rehman, et al. *Am. J. Physiol.*, 272:H190-H1971, 1997). Briefly, the rats were anesthetized by intraperitoneal injection (35mg/kg) of sodium pentobarbital (Anpro Pharmaceuticals). Anesthesia was maintained during the course of the experimental protocol (2-3 hrs) by subsequent injection of pentobarbital (5-10 mg/kg) every 45-60 minutes. Animals were placed in the supine position and the bladder and prostate were exposed through a midline abdominal incision. The inferior hypogastric plexus (i.e., the pelvic plexus or major pelvic ganglia), pelvic nerves and the cavernous nerve were identified posterolateral to the prostate on both sides, and the stainless-steel bipolar wire electrodes were placed around these structures for electrical stimulation. The penis was denuded of skin, both crura were exposed by removing part of the overlying ischiocavernous muscle. In order

to monitor intracavernous pressure (ICP), a 23-gauge cannula was filled with 250 U/ml of heparin solution, connected to PE-50 tubing (Intramedic, Becton Dickinson) and inserted into the right corpus cavernosum. The tubing was then fixed to the tunica with a 7-0 Dermal on suture, to ensure stability during measurement of ICP. Another 23 gauge cannula was connected to a 1 ml syringe and inserted into the left corpus cavernosum for intracavernous drug injection. Systemic arterial blood pressure (BP) was monitored via a 25-gauge cannula placed into the carotid artery.

Both pressure lines (BP) and (ICP) were connected to a pressure transducer, which was, in turn, connected via a Transducer amplifier (ETH 400 CV Sciences Inc.) to a data acquisition board (Mac Lab/8e, ADI instruments, MA). Real-time display and recording of pressure measurements was performed on a Macintosh computer (Mac Lab software V3.4). The pressure transducers and A/D board were calibrated in cm of H_20.

Neurostimulation of cavernous nerve and recording of intracavernosal pressure. Direct electrostimulation of the cavernous nerve was performed with a delicate stainless-steel bipolar hook electrode attached to the multijointed clamp. Each probe was 0.2 mM in diameter; the two poles were separated by 1 mM. Monophasic rectangular pulses were delivered by a signal generator (custom made and with built- in constant current amplifier). Stimulation parameters were as follows: frequency: 20 Hz, pulse width; 0.22 msec, duration; 1 minute. The current protocol was the application of increasing current at the following intervals: 0.5, 1, 2, 4, 6, 8 and 10 mA. The changes in intracavernous pressure and systemic blood pressure were recorded at each level of neurostimulation.

RNA preparation. Total RNA was extracted from frozen tissue using the TRIzol method. Briefly, tissue was homogenized in TRIzol reagent by a Polytron homogenizer (Brinkman, NY) for about 30 seconds. The tissue lysate was then transferred to a polypropylene, 10 x 1.8 cm round bottom tube (Falcon, Becton Dickinson, New Jersey), and incubated for 5 minutes at room temperature. Then chloroform was added and the solution was centrifuged at 12,000 x g, 4°C for 15 minutes. RNA was extracted in the aqueous phase from the TRIzol and chloroform

mixture and precipitated from the aqueous phase by mixing with isopropyl alcohol and centrifuged at 12,000 x g for 10 minutes at 4°C. RNA was stored in 0.08 M sodium acetate and 70% ethanol.

Northern blot analysis. Twenty μg of total RNA from each tissue sample was electrophoresed in 1% agarose containing 2.2M formaldehyde and transferred onto nylon membranes by capillary transblotting. The position of 28s and 18s ribosomal RNA bands on the ethidium-stained gels were observed under ultraviolet illumination before transblotting. RNA was fixed to the filter by heating for 2 hours at 80°C. The hSlo cDNA was cut from the XhoI and XbaI sites of pcDNA and purified from an agarose gel, random primer biotin labeled with the NEBlot Phototype Kit (New England Biolabs) and used as cDNA probes for Northern blots. Hybridization was carried out in Rapid-hyb buffer (Amersham, Arlington Heights, IN) at 68°C for 2 hours. Filters were washed two times in 2.5X SSC and 0.1% SDS at room temperature, followed by two washes in 1X SSC and three washes in 0.1% SDS at 68°C for twenty minutes each time. Following the washing, the membranes underwent detection steps using strepavidin and Biotin Alkaline Phosphotase with CDP-Star substrate according to the manufacturer's instructions (New England Biolabs). After incubation with CDP-Star substrate, the membranes were removed and exposed to the Hyperfilm (Amersham) in an intensifying screen for at least fifteen minutes. The film was developed with time adjustments and the bands were analyzed.

RT-PCR. To further confirm and detect expression of the recombinant K_{Ca}, two distinct PCR strategies were used: 1. with primers specific to the plasmid sequences (T7 and SP6 promoters), and 2. with primers specific for the 5'-untranslated region (see below). With respect to the latter, oligonucleotide primers for PCR amplification of the 5'-untranslated region (approximately 0.14 kb) were 5'-GCCGCCACCATTTGCCAT-3' (3' primer; coding for the first six amino acids of K_{Ca}) and 5'-CCCTATAGTGAGTCGTATTA-3' (5' primer; specific to the T7 promoter). With respect to the former, oligonucleotide primers for PCR amplification of the full K_{Ca} insert (approximately 4.2 kb) were T7 promoter region (see above) and the SP6 promoter region (5'-CTAGCATTTAGGTGACACTATAG-3').

The primers for an endogenous K_{Ca} region (base 909-1074; 5'-GCTCTCCATATTTATCAGCAC-3' and 5'-AACATCCCCATAACCAAC-3') were used as a control.

The RT-PCR was performed using the Superscript One-Step RT-PCR System (Gibco BRL, Grand Island, NY) with a final volume of 50 μl. The RT-PCR mixture included a buffer containing 1 μg of total RNA, 1 μM of the sense and antisense oligonucleotide primers, 0.4 mM of each dNTP, Superscript II RT/*Taq* Mix, 80 units of $RN_{ASE}OUT$ recombinant ribonuclease inhibitor (Gibco BRL), and an optimized concentration of $MgSO_4$ as per manufacturer's instructions. First-strand cDNA was performed at 45°C for 30 minutes and denatured at 94°C for 5 minutes. The PCR was performed with the cycle of 45°C annealing for 1 minute, 72°C extension for 1.5 minutes, and 94°C denaturing for 1 minute, for a total of 35 cycles with a final extension step for 10 minutes. For amplification of the full insert, one μl of eLONGase was added to the reaction mixture at 68°C for 5 minutes for extension.

Statistical Analysis. All statistical analyses were performed using the Stat-View 4.5 software (Abacus Concepts, Berkeley, CA). Either a Two-Way analysis of variance (ANOVA) with a post-hoc multiple comparisons (Tukey) or a two-tailed Student's t test for unrelated samples was utilized, as appropriate for comparison of group means for parameters of interest between gene therapy rats (i.e., maxi-K transfected), age-matched control animals, and the young control rats. All differences were considered significant at $p<0.05$. Unless otherwise stated, all data are expressed as the mean (±S.E.M.).

Construction of stimulus-response curves. Stimulus-response curves were generated for the effects of neurostimulation on intracavernous pressure by expressing the change in intracorporal pressure as a fraction of the mean systemic blood pressure (expressed in ICP/BP) and plotting this fraction as a function of the magnitude of neurostimulation (1,2,4,6,8,10 mA). All data were plotted using Sigma Plot software for the Macintosh (Sigma Plot Mac V5.0 Jandel Scientific, San Rafael, CA).

B. Bladder Dysfunction Gene Therapy Experiments Using Naked DNA

Injection of Lac Z DNA and Maxi-K DNA. Lac Z DNA was prepared as described above. Briefly, transfer of Lac Z DNA (coding for β-galactosidase) into rat bladder smooth muscle was accomplished by injecting the plasmid pCMVβ as naked DNA into the bladder as follows. An abdominal incision was made in the pentobarbitol anesthetized rat (sodium pentobarbitol, 35 mg/kg), and the bladder was exposed. 65 μg of Lac Z DNA was injected into the bladder in 1 ml of buffer solution, with an insulin syringe. The needle opening was cauterized, and the abdominal musculature was closed with sutures and the skin was closed with surgical staples. The animal was injected, and 4 days later the animal was sacrificed. The bladder was quickly excised and placed in 4% paraformaldehyde solution (4 hours), and transferred to the chromogenic solution (described above). The bladder was immediately placed in an incubator at 37°C. Fourteen hours later, the bladder tissue was removed from the incubator and photographed. 100 μg of maxi-K pcDNA was prepared as described above and injected into the bladder of four rats. Incorporation of maxi-K pcDNA into the bladder smooth muscle cells was shown by Southern blot analysis.

II. Results

A. Erectile Dysfunction Gene Therapy Experiments Using a Recombinant Vector

Potassium channels and corporal smooth muscle function: Evidence that altering K channel function can increase "sensitivity" to relaxation. Recent studies by the inventors have indicated that hyperpolarization of corporal smooth muscle cells via activation of potassium channels represents an important mechanism for controlling corporal smooth muscle tone (Holmquist, F., et al., *J. Urol.* 144 (1990) 146; Christ, G.J., et al., *Journal of Andrology* 14.5 (1993) 319-28; Fan, S.F., et al., *J. Urol.* 153 (1995) 818; Christ, G.J., *Urological Clinics of North America* 22.4 (1995) 727-745). This observation reflects the fact that sustained contraction of human corporal smooth muscle, which is characteristic of flaccidity (the condition the vast majority of the time) is largely dependent on continuous transmembrane

Ca^{2+} flux through voltage-gated Ca^{2+} channels. The activity of these voltage dependent calcium channels in corporal smooth muscle cells is, in turn, closely modulated by hyperpolarizing currents, initiated and carried mainly by K channels. Among the subtypes of K^+ channels, the ≈180 pS Ca^{2+} sensitive maxi-K channel is one of the most prominent in corporal smooth muscle cells (Fan, S.F., et al., *J. Urol.* 153 (1995) 818). Membrane hyperpolarization of corporal smooth muscle cells following activation of K channels can be accomplished by both receptor (e.g., PGE or NO) and nonreceptor-mediated (e.g., NO or cGMP) stimuli, derived from neuronal as well as endothelial sources. These data are summarized in Figures 14A-14B and Table 5.

The putative mechanism of action is thought to be as follows: release of an endogenous corporal vasorelaxant(s), for example, nitric oxide, is thought to either directly activate the K channel, or, to regulate K channel activity secondary to activation of soluble guanylate cyclase, increases in intracellular cGMP levels, activation of G kinase, and phosphorylation of cellular proteins, including nonjunctional ion channels such as K and Ca^{2+} channels. Increased kinase activity (either A or G) has an opposing action on Ca^{2+} and K channels, resulting in decreased activity of the former and increased activity of the latter. Thus, an elevation in intracellular NO and/or cGMP levels can result in both activation of K channels and inhibition of Ca^{2+} channels. The algebraic sum of these two opposing effects is to significantly diminish transmembrane calcium flux, resulting in diminished corporal smooth muscle tone, and thus, corporal smooth muscle relaxation.

Since the activity of the maxi-K channel appears to be modulated by all of the physiologically relevant endogenous regulators of corporal smooth muscle tone (Figure 2), including PGE (Zhang, et al., *Journal of Urology*, 155:678A (1996); Zhao, et al., *J. Urol.* 154 (1995) 1571-1579; Zhao, et al., *Journal of Urology*, 155:678A (1996) as well as NO (Christ, et al., unpublished observations), it is clearly an important final common mediator of the degree of corporal smooth muscle tone. Consistent with this hypothesis, the inventors have preliminary evidence that altered regulation/function of this channel may represent an

important feature of the presence of organic erectile dysfunction per se, in human corporal smooth muscle (Fan, S.F., et al., *J. Urol.* 153 (1995) 818; Christ, G.J., *Urologic Clinics of North America*, 22.4:727-745 (1995); Christ, G.J., et al., *Journal of Urology*, 155:620A (1996)). For all of these reasons, the inventors feel that the relatively stable transfection of corporal smooth muscle cells with the human smooth muscle maxi-K channel cDNA represents an important and attractive strategy for modulating erectile capacity.

Nitric oxide and corporal smooth muscle function: Evidence that increasing the amount of NO expressed in corporal tissue can increase the "driving force" for relaxation. An abundance of recent experimental evidence documents the important role played by nitric oxide in arterial and corporal smooth muscle relaxation, and thus, penile erection (Argiolas, et al., *Neuropharmacology* 33.11 (1994) 1339-44; Burnett, et al., *Science* 257.5068 (1992) 401-3; Trigo-Rocha, et al., *J. of Physiology* 264.2 Pt 2 (1993) H419-22; Burnett, et al. *Biology of Reproduction* 52.3 (1995) 485-9). For example, electrical stimulation of both human (Saenz de Tejada, et al., *New England Journal of Medicine* 320.16 (1989) 1025-30) and rabbit (Ignarro, et al., *Biochem Biophys Res Commun* 170 (1990) 843-850) corpus cavernosal muscle strips results in smooth muscle relaxation. These responses are thought to be mediated via release of nitric oxide. Consistent with this supposition, these relaxation responses can inhibited by nitroglycerin-substituted analogs of L-arginine, which block NO formation (Ignarro, et al., *Biochem Biophys Res Commun* 170 (1990) 843-850; Holmquist, et al., *Acta Physiol Scand* 141 (1991) 441-442; Kim, et al., *J. Clin. Invest.* 88 (1991) 112-118). Further, the relaxation of both rabbit and human corporeal smooth muscle can be induced through compounds that release NO (Ignarro, et al., *Biochem Biophys Res Commun* 170 (1990) 843-850; Rajfer, et al., *New England Journal of Medicine* 326 (1992) 90-94; Christ, G.J., et al. *Urological Clinics of North America* 22.4 (1995) 727-745). Moreover, the importance of the NO dependent pathway for relaxation of human corporal smooth muscle has also been documented (Bush, et al., *J. Urol.* 147 (1992) 1650-1655; Trigo-Rocha, et al., *Neurology & Urodynamics* 13.1 (1994) 71-80; Christ, et al., *Canadian Journal of*

Physiology and Pharmacology, 73:714-726 (1995)).

Nitric oxide is produced by the enzyme nitric oxide synthase (NOS) as a product of the enzymatic conversion of L-arginine to L-citrulline. NO is produced in endothelial cells upon cholinergic stimulation or by neuronal sources (released from NANC nerve terminals). With respect to the latter, NO is a novel neurotransmitter in that it is not stored in synaptic vesicles in nerve terminals but is synthesized on demand. Biochemical and histochemical evidence in rabbit and rat penis suggests that the NOS isozyme which functions in penile erection belongs to the cNOS type (Burnett, et al., *Science* 257.5068 (1992) 401-3). A neural source for NOS in the rat penis was demonstrated by Keast (Keast, J.R., *Neurosciences Letter* 143 (1992) 69-73; Burnett, et al., *J. Urol.* 150.1 (1993) 73-6; Burnett, et al., *Science* 257.5068 (1992) 401-3) localizing NOS to the autonomic nerves of rat and human penis by both immunohistochemistry with rat cNOS antibody and by NADPH diaphorase histochemistry. The mechanism of action of NO is thought to be as follows: after production, NO, a highly lipophilic substance, quickly diffuses (in three dimensions (Christ, et al., *Biophysical Journal* 67:1335-1344 (1994)) into corporal smooth muscle cells, where it results in activation of soluble guanylate cyclase, catalyzing the conversion of GTP to cGMP. This increase in cGMP activates protein kinase G, which, as illustrated in Figure 2, leads to decreases in intracellular Ca^{2+}, producing corporal smooth muscle relaxation (Moncada, S., *Acta Physiol Scand* 145 (1992) 201-227). As mentioned above, there is also recent evidence that in at least some vascular smooth muscle cells, NO may directly interact with K channels to elicit hyperpolarization and smooth muscle relaxation.

In the flaccid state NOS activity is thought to be minimal (Ignarro, et al., *Biochem Biophys Res Commun* 170 (1990) 843-850; Rajfer, et al., *New England Journal of Medicine* 326 (1992) 90-94; Azadozi, et al., *J. Urol.* 147.1 (1992) 220-225; Brock, et al., *Urology* 42.3 (1993) 412-417; Hellstrom, et al., *J. Urol.* 151.6 (1994) 1723-7; Pickard, et al., *British Journal of Urology* 75.4 (1995) 516-22; Carrier, et al., *J. Urol.* 153.5 (1995) 1722-7; Garban, et al., *American Journal of Physiology* (1995) H467-H475; Burnett, et al., *Biology of Reproduction* 52.3 (1995)

485-9). The intensity of the histochemical detection of NADPH diaphorase in cavernosal tissue has been shown to be decreased in patients with cavernosal nerve injury, implying decreased NOS activity (Brock, et al., *Urology* 42.3 (1993) 412-417). Furthermore, it has been suggested that the impaired relaxation responses (to electric field stimulation) of the cavernosal nerve of diabetic men may also be due to decreased NOS production (Saenz de Tejada, I., *New England Journal of Medicine* 320.16 (1989) 1025-30; Taub, et al., *Urology* 42 (1993) 698; Christ, G.J., *Urologic Clinics of North America* 4 22.4 (1995) 727-745; Vernet, et al., *Endocrinology* 136 (1995) 5709-5717). Thus, the introduction of a constitutively expressed cDNA for nitric oxide synthase might be expected to result in corporal smooth muscle relaxation and greater resting and nerve-stimulated pressure responses. As described below, the cDNA for neuronal NOS has been inserted into the corpus cavernosa of rats and a statistically significant, and physiologically relevant alteration was observed in the intracavernous pressure response to electrical stimulation of the cavernous nerve (see Table 2 below).

Table 2: Intracavernous pressure (ICP) response following nerve stimulation in NOS and control group

	Cavernous Nerve Stimulation*							
	ICP♣ (Basal)	ICP♦ 0.5mA♥ M±SEM♠	ICP 1mA M±SEM	ICP 2mA M±SEM	ICP 4mA M±SEM	ICP 6mA M±SEM	ICP 8mA M±SEM	ICP 10mA M±SEM
NOS gene therapy (n=12)	14.62 ± .64	60.02 ± 5.9	102.50 ± 3.89	110.49 ± 4.65	113.93 ± 2.17	117.72 ± 2.80	121.26 ± 3.26	129.03 ± 5.4
Age-Matched Control received vehicle only (n=8)	8.1 ± .37	45.81 ± 3.63	75.242 ± 3.59	83.93 ± 3.23	83.59 ± 4.52	85.67 ± 3.75	87.94 ± 4.45	92.23 ± 3.62
P value	.0001	.0085	.0001	.0001	.0001	.0001	.0001	.0003

* Cavernous nerve stimulation performed on both sides (Observation x 2 the # of rats)

♦ ICP-Intracorporal pressure in cm of H_2O

♣ BCP-Basal corporal pressure in cm of H_2O before neurostimulation

♥ mA-milli Amperes of stimulus to the nerve

♠ M±SEM-Mean and standard error of the mean

Selection of Rat model. The rat was selected for the gene therapy studies, as the rat penis has been shown to be histologically and pharmacologically similar to human penis (Lesson, et al., *Investigative Urology* 3.2 (1965) 144-145). Among many known models, the rat is excellent for the study of penile erection (Lesson, et al., *Investigative Urology* 3.2 (1965) 144-145; Quinlan, et al., *J. Urol.* 141.3 (1989) 656-61; Chen, et al., *J. Urol.* 147 (1992) 1124-1128; Martinez-Pineiro, et al., *European Urology*, 25 (1994) 62-70) neurogenic and diabetic impotence (Rehman, et al., *Am J Physiol* (1997) 41:H1960-H1971).

Maxi-K Channel Results: Evidence that K Channels Modulate Corporal Smooth Muscle Relaxation. The putative role of the maxi-K channel in mediating NTG- and PGE_1-induced relaxation responses in the human corpora. Evidence from both cellular and isolated tissue studies documents the important role of the maxi-K channel in modulating relaxation responses in this tissue.

NTG & maxi-K: As illustrated by the representative example in Figure 1, in addition to the aforementioned effects on PE-induced contractile responses, selective blockade of the maxi-K channel with 1 mM TEA also results in a significant attenuation of the NTG-induced relaxation response (100 nM). Studies on five other corporal tissue strips revealed that the mean ±S.E.M. %relaxation response elicited by 100 nM NTG was of 20.3 ± 3.2%; compare this value with the expected value of 50.1%, as determined in another recent publication. This finding documents that activation of the maxi-K channel is also likely to be an important component of the NTG-induced relaxation response. Consistent with this hypothesis, preliminary patch clamp studies cells (i.e., attached patch recording mode) on cultured corporal smooth muscle cells, with albeit much greater NTG concentrations (100 μM), demonstrated an NTG-induced increase in maxi-K channel activity to cultured corporal smooth muscle.

PGE_1 & maxi-K: Recent electrophysiological studies utilizing all four recording modes documented that PGE_1 causes a concentration-dependent increase in the activity of the maxi-K channel; over the same concentration range as the inventors observed cAMP formation in cultured cells and relaxation of precontracted isolated human corporal smooth muscle strips (Zhang, et al., *J. Urol.* 155:678A (1996)). In addition, this increase in maxi-K channel activity is correlated with significant alterations in the ET-1-induced intracellular calcium transient seen in fura-2 loaded cultured corporal smooth muscle cells. Specifically, preincubation of cultured human corporal smooth muscle cells with 500 nM PGE_1 resulted in a significant ≈40% decrease in the peak amplitude of the ET-1-induced (50 nM) calcium transient above baseline (i.e., ≈70 nM) from a control value of 161.5 ± 19.5 nM to 102.6 ± 9.5 nM (Zhao, et al., *J. Urol.* 155:678A (1996)). Note that this decrease is indistinguishable from the decrease seen in absence of

extracellular Ca^{2+} (2 mM EGTA) or when cells are preincubated with nifedipine (Zhao & Christ, *J. Urol.* 154:1571-1579 (1995)) (or verapamil (Christ et al., unpublished observations); both of which are blockers of the L-type voltage-dependent calcium channel).

Table 3. Summary of Documented *in vitro* effects of the maxi-K channel on the physiology of corporal smooth muscle

PE-induced contraction increased	Resting corporal smooth muscle tone increased	Resting intra-cellular calcium levels decreased	ET-1-induced calcium response decreased	Relaxation response decreased	Channel Activity increased
YES (1 mM TEA)	YES (10-100 mM TEA)	YES transfect-ion with (hSlo cDNA)	YES transfection with (hSlo cDNA) or preincubation with PGE_1 (500 nM)	YES (1 mM TEA)	YES 100 μM NTG or 30 nM-30μM PGE_1

Finally, it is herein shown (Table 5) that transfection of cultured human corporal smooth muscle cells with the human smooth muscle maxi-K cDNA, hSlo, results in a significant decrease (≈25%) in the mean resting intracellular calcium levels, as well as a decrease (≈45%) in the peak amplitude of the ET-1-induced intracellular calcium transient. While the physiological significance of the former observation is uncertain, note, that the effects of transfection on the ET-1-induced intracellular calcium transient (Table 5), are remarkably similar to those seen in the presence of PGE_1, nifedipine and verapamil, or in the absence of extracellular calcium. Taken together, all of these data are totally consistent with the hypothesis that increased maxi-K channel activity, following either the presence of a greater number of maxi-K channels (presumably the effect of the transfection, although this has not yet been unequivocally established), or following cellular activation with, for example, PGE_1, is associated with cellular hyperpolarization, decreased transmembrane calcium flux through L-type voltage- dependent calcium channels, and a corresponding decrease in the peak amplitude of the ET-1-induced

calcium transient. Moreover, since the peak amplitude of the intracellular calcium transient measured on cultured corporal smooth muscle cells accurately tracks the magnitude of the steady-state contractile response measured on isolated corporal tissue strips (Christ, et al., *J. Urol.* 153:1998-2003 (1995)), these data provide compelling evidence that the increased activity of the maxi-K channel modulates the magnitude of the corporal smooth muscle contractile response, at least in part, by altering transmembrane calcium flux. It is important to point out that the dual role of the maxi-K channel in modulating both the magnitude of contraction and relaxation takes advantage of the fact that maxi-K-induced inhibition of transmembrane calcium flux can occur following agonist (PE or ET-1)-induced increases in intracellular calcium, or following PGE_1- (presumably PKA) or NTG-induced (presumably PKG) increases in phosphorylation of the maxi-K channel; these ideas are consistent with the literature in other vascular smooth muscle cell types (*see* Table 4).

Table 4. Effects of vasoactive compounds on ion channel activity, membrane potential and corporal smooth muscle tone

	Membrane Potential *-30mV* *(DEPOLARIZED; i.e., Contracted)*		
Agonist	Channel Type Affected	Putative Mechanism	Effect on Smooth Muscle Tone
ET-1	L-type Ca^{2+} increases	voltage or phosphorylation	increased tone
PE	K_{Ca} increases	Ca^{2+} -sensitive	modulates increase in tone
KCl	K_{ATP} increases	decreased ATP	modulates increase in tone
TEA	K_{Ca} decreases	channel blockade	increased tone
Glibenclamide	K_{ATP} decreases	channel blockade	increased tone
	⇑ -40 to -50 mV (RESTING POTENTIAL) ⇓		
PGE_1	K_{Ca} increases	phosphorylation	decreased tone
NTG	L-type Ca^{2+} decreases	voltage or phosphorylation	decreased tone
PINACIDIL	K_{ATP} increases	increased mean open time	decreased tone
	Membrane Potential *-60 mV* *(HYPERPOLARIZED; i.e., Relaxed)*		

Table 5. Effect of transfection with hSlo cDNA on resting and ET-1-induced changes in Ca_i.

Control Cells (n=17)		TT Transfected Cells (n=32)	
Resting Ca_i	ET-1 (50 nM)	Resting Ca_i	ET-1 (50 nM)
74.7±4.0 nM	164.1±17.8 nM	*56.7±2.5 nM	*90.8±6.6 nM

* -denotes a statistically significant difference from control values; $p<0.001$, Student's t test for unpaired samples. Ca_i refers to the intracellular calcium concentrations. The values given for the ET-1 induced increase represent the peak amplitude of the intracellular calcium transient as described in Zhao & Christ, *J. Urol.* 154 (1995) 1571-1579.

As a first test of the potential utility of modulating K channel activity to the therapy of erectile dysfunction, preliminary studies were conducted on a rat model *in vivo*. Briefly, it was found that injection of naked cDNA encoding the human smooth muscle maxi-K channel (i.e., hSlo; obtained from Dr. Salkoff, Washington University), into the rat corpora, resulted in significant uptake and gene expression. This was documented by the fact that the nerve-stimulated intracavernous pressure increases observed on 8 month old Sprague-Dawley rats injected with the maxi-K cDNA were significantly greater than the intracavernous pressure responses seen in age-matched control, sham-operated animals (Figure 9 and Table 3). Moreover, the incorporation of this gene (again, as judged by the significant elevation of intracavernous pressure relative to control animals) remained stable for more than three months. These *in vivo* studies are entirely consistent with all of the *in vitro* observations made by the inventors, and thus, further document the importance of K channels to the modulation of corporal smooth muscle tone.

B. Results for Erectile Dysfunction Gene Therapy Experiments Using Naked DNA

Demographics of Experimental Animals. A total of 74 male Sprague Dawley rats were used in these studies (Taconic Farms, Germantown, NY). 29 of these animals were 10-12 weeks old, and 5 of these served as the young control animals. The remaining 45 animals were purchased as retired breeders. They were all greater than 9 months old and ranged in weight from 500-700g. All rats were fed Purina lab rodent chow *ad libitum* and housed individually with a 07.00-19.00 light cycle. The demographics of the animal population, with respect to protocol enrollment are displayed in Table 4.

Table 6: Mean Intracavernous Pressure (ICP) and Blood Pressure (BP) measurements made during cavernous nerve stimulation

	Maxi-K (N=17)		Age-Matched Control (N=12)		Young Control (N=5)	
Neurost-imulation (mA)	ICP (cmH_2O)	BP (cmH_2O)	ICP (cmH_2O)	BP (cmH_2O)	ICP (cmH_2O)	BP (cmH_2O)
0.5	111 ± 7 (n=6)	198 ± 6	43 ± 11 (n=5)	178 ± 12	102 ± 16 (n=5)	183 ± 16
1.0	107 ± 7 (n=16)	192 ± 5	55 ± 5 (n=12)	178 ± 6	132 ± 10 (n=5)	180 ± 10
2.0	114 ± 11 (n=16)	198 ± 5	63 ± 5 (n=12)	177 ± 7	133 ± 10 (n=5)	180 ± 10
4.0	121 ± 7 (n=15)	196 ± 5	57 ± 5 (n=12)	177 ± 7	133 ± 10 (n=5)	186 ± 10
6.0	124 ± 5 (n=11)	190 ± 6	53 ± 6 (n=9)	180 ± 9	131 ± 13 (n=3)	181 ± 10
8.0	129 ± 5 (n=8)	191 ± 5	59 ± 7 (n=9)	181 ± 9	133 ± 11 (n=4)	184 ± 11
10.0	142 ± 6 (n=7)	195 ± 5	56 ± 13 (n=4)	191 ± 7	132 ± 11 (n=4)	180 ± 11

Transfection of pCMVβ/Lac Z into rat corpora *in vivo*. Twelve rats were injected with pCMVβ/Lac Z and followed for up to ≈11 weeks (75 days) post injection, with an equivalent number of age-matched control rats run in parallel (see Table 4). Histological evaluation of corporal tissue excised from rats receiving an intracorporal injection of naked Lac Z DNA revealed the presence of significant chromogenic material in 10/12 animals. Figure 7A shows a representative example of the presence of chromogenic material in the corpora of a rat 60 days post injection. Thus, incorporation of naked DNA appears sustained, over this time period, in this tissue. Similarly prolonged incorporation of gene transfer products has been reported in other vascular tissues.

Resting and neurostimulation-induced intracavernous pressure responses in maxi-K transfected and sham-operated control rats. A One-Way analysis of variance revealed that there was a significant difference in the resting intracavernous pressure in rats transfected with the pcDNA/*hSlo* DNA. There was no detectable difference in the mean resting intracavernous pressure or mean arterial pressure values among the three treatment groups ($P>0.07$; One-Way ANOVA). the mean ± S.E.M. values for ICP and BP, respectively, were for the pcDNA/*hSlo* DNA rats (N=17): 22.7 ± 1.9 and 177.4 ± 4.3 cmH_2O, for the age-matches control animals (N=12) 14.9 ± 3.3 and 177 ± 4.5 cmH_2O, and for the young control animals (N = 5) these values were 19.8 ± 3.3 and 168.6 ± 7.4 cmH_2O.

The effects of current stimulation of the cavernous nerve on the intracavernous pressure response in rats *in vivo* was utilized to evaluate the potential physiological relevance of over-expression of the maxi-K channel following intracavernous injection of the pcDNA/hSlo DNA (see Methods). For these studies, the rats were divided into distinct treatment groups. All animals were examined using identical neurostimulation protocols with the magnitude of current stimulation ranging from 0.5 mA. Representative responses to current stimulation in pcDNA/hSlo transfected, age-matched control and young control animals are illustrated in Figure 9 in response to 2 mA current stimulation.

For statistical comparison of treatment effects, the mean amplitude of the intracavernous pressure response at each level of current stimulation was expressed as a fraction of the mean arterial blood pressure (ICP/BP) during current stimulation. A Two-way analysis of variance revealed that there was a significant effect of treatment ($p<0.001$ on the mean ICP/BP, but no effect of current stimulation ($p>0.13$), and no treatment-current stimulation interaction ($p=0.26$). The mean response for all animals in each treatment group is graphically represented in Figure 9, and the data are summarized in Table 5. Although post-hoc analysis documented a significant difference in the mean amplitude of the intracavernous pressure response to all levels of current stimulation between the young control group and the maxi-K transfected groups

($p<0.05$), both groups had significantly elevated intracavernous pressure responses compared to the age-matched control animals ($p<0.05$).

In addition, the pcDNA/hSlo transfected animals were further subdivided according to whether the *in vivo* studies were conducted 1 month, 2 months, 3 months or 4 months post-transfection, with age-matched control animals run in parallel. Consistent with the overall trend for the entire animal population (see Figure 4), Two-way analysis of variance revealed that intracorporal injection of the pcDNA/hSlo DNA was associated with significantly elevated intracorporal pressures at all levels of current stimulation for both the 1 month (Figure 10A) and 2 month (Figure 10B) post injection time points. Statistically meaningful conclusions could not be drawn from the small number of observations at the 3 month and 4 month post injection time points. However, Figure 10C does illustrate that apparently qualitatively similar results were obtained.

Incorporation of recombinant human K_{Ca} into rat corporal smooth muscle. The gene expression level of the recombinant human K_{Ca} in the transfected rat corporal smooth muscle was examined by both RT-PCR and Northern blot analyses. Total RNA from pcD-NA/K_{Ca}-transfected, pcDNA-transfected control tissue were RT-PCR amplified with primers as described in the Methods section and displayed in Figures 11A-11C. As illustrated, the amplification of the 5' untranslated region resulted in a significant cDNA band from the pcDNA/K_{Ca}-transfected (molecular size is approximately 4.2 kb) was also RT-PCR amplified from the pcDNA/K_{Ca}-transfected tissue, but not from the pcDNA-transfected control (Figure 11C). The quality of the RNA from all tissues was further examined with primers that amplified the endogenous K_{Ca}. As indicated, the endogenous K_{Ca} was amplified to a comparable level in all tissues (Figure 11B). The presence of the recombinant human K_{Ca} expression was further examined by Northern blotting with an *hSlo* insert as a probe (Figure 12). Once again, RNA from the pcDNA/K_{Ca}-transfected tissue, but not from the pcDNA-transfected or control tissue expressed a detectable level of the recombinant human K_{Ca}. The recombinant human K_{Ca} band was detected between the 18S and 28S RNA bands, with an approximate molecular size of 4.2kb. In light of the high stringency conditions used in this

assay (see Materials & Methods), it is not surprising that the endogenous K_{Ca} was not detected.

III. DISCUSSION

Many recent advances have been made in the application of gene therapy to the treatment of human disease. In particular, techniques for gene transfer into vascular smooth muscle cells have been developed in the hope of providing a novel therapeutic strategy for the treatment of several cardiovascular diseases. Among these are atherosclerosis, vasculitis and restenosis after balloon angioplasty. Such studies have provided important information on the efficiency and persistence of gene transfer methods in smooth muscle cells.

In light of these seminal observations, it was the explicit aim of these studies to begin to evaluate the feasibility of somatic gene transfer into corporal smooth muscle as a novel therapeutic strategy for the treatment of erectile dysfunction. The rationale for this approach is related to the fact that the tone of the corporal smooth muscle cells in the specialized vascular tissue of the penis plays a critical role in modulating the flow of blood to and from the penis, and thus in determining erectile capacity. This fact makes them a logical target for molecular intervention in the treatment of erectile dysfunction. Furthermore, given the central role of the maxi-K channel in modulating human corporal smooth muscle tome, the inventors examined the physiological impact of transfection of corporal smooth muscle with hSlo cDNA in a rat model *in vivo*.

The major findings are as follows. First, after a single intracorporal injection of naked pCMVβ/Lac Z DNA (see Methods) expression of β-galactosidase activity is sustained for at least 75 days. This clearly demonstrates that in the rat corpora, *in vivo*, relatively prolonged expression of extrachromosomal genes that encode physiologically detectable protein products is feasible (Figures 7A and 7B). Consistent with these observations, the mean amplitude of the nerve-stimulated intracavernous pressure response is significantly augmented over a similar time course in rats, *in vivo*, following a single intracavernous injection of naked pcDNA/hSlo DNA (see Figures 8-10). Moreover, both RT-PCR techniques and

Northern blots revealed that, at least for the two month time point, the observed augmentation in the nerve-stimulated intracavernous pressure response is correlated with increased expression of the *hSlo* mRNA (Figures 11 & 12). Presumably, the same holds true for the longer time points (i.e., 3-4 months), although this was not directly evaluated in this series of experiments. Moreover, there was no detectable effect of vector alone or sham surgery (see Methods) on the nerve-stimulated intracavernous pressure responses at any time point examined in these initial studies (see Figures 8-10). Taken together, these data provide compelling evidence that the enhancement in the nerve-stimulated intracavernous pressure responses observed in the maxi-K transfected animals, relative to the age-matched control animals, is most certainly related to the extrachromosomal expression of the hSlo cDNA, and a nominally corresponding increase in expression of the maxi-K channel protein.

In order to better judge the physiological meaning of the increase in ICP in the maxi-K transfected animals, a second group of young control (2-3 months old) animals was also studied. As illustrated in Figure 8, the increase in the mean amplitude of the nerve-stimulated ICP/BP ration in the 10-13 month old maxi-K transfected animals, approximates, but does not exceed, the response observed in the adolescent rats. This would suggest that the putative increased expression of the maxi-K channel in the "older" animals is associated with a nerve-stimulated ICP response that is nominally equivalent to the best response expected under "normal" physiological conditions in younger animals.

With respect to the mechanistic basis for the inventors' observations, it is clear that the injected hSlo cDNA is likely to be taken up into all cell types present in the rat corpora. In this regard, despite the fact that a role for uptake of the hSlo cDNA in the endothelial cell in mediating the observed increases in nerve-stimulated intracavernous pressure cannot be unequivocally excluded, the discussion is confined to putative effects resulting from uptake and expression in the corporal smooth muscle cell. This seems reasonable in light of the fact that the corporal smooth muscle cell makes up the vast majority of the corporal parenchyma, and moreover, the relaxation of the corporal smooth muscle is both

necessary and sufficient for erection. A more precise analysis of the cellular disposition of the *hSlo* cDNA and the resulting expression of the α-subunit of the maxi-K channel, as well as the relative percentage of cells thus affected, will necessarily be the province of future investigations.

Therefore, although a cause-effect relationship was not established, presumably, the mechanistic basis for the increased maxi-K channel activity would be related to the commensurate augmentation in the hyperpolarizing ability of the corporal smooth muscle cells. Moreover, given the exquisite dependence of sustained corporal smooth muscle contraction on continuous transmembrane calcium flux, it stands to reason that the increase in hyperpolarization is associated with a decreased transmembrane calcium flux through L-type voltage-dependent calcium channels, and a corresponding decrease in the free intracellular calcium concentration; ultimately promoting greater corporal smooth muscle relaxation. Thus, increasing the expression of the maxi-K channel would logically dictate an increased sensitivity of the smooth muscle cells to the same level of neural stimulation.

As with all other *in vivo* gene therapy approaches, the potential utility of this genetic technique to the treatment of human erectile dysfunction heralds the following two considerations: 1. What is the likelihood of affecting only the desire cell type(s), and 2. What percentage of target cells must be affected in order to see a physiologically relevant therapeutic effect. In light of such considerations, there are two main reasons for suspecting that gene therapy of erectile dysfunction may be inherently more successful than its proposed uses in other, more systemic, cardiovascular disorders, such as atherosclerosis, vasculitis and restenosis after balloon angioplasty.

First, it is a well documented fact that the corporal smooth muscle cells are interconnected by a ubiquitously distributed population of intercellular channels known as gap junction proteins, with connexin 43 as the predominant isoform expressed in the human penis. These intercellular channels provide partial cytoplasmic continuity between adjacent smooth muscle cells, allowing the intercellular exchange of physiologically relevant ions (K^+ and Ca^{2+}) and second

messenger molecules (IP3, cAMP, cGMP). As such, the presence of gap junctions in the rat and human provides an important anatomic substrate for coordinating the syncytial contraction and relaxation responses that are a prerequisite to normal penile erection and detumescence. That is, intercellular communication among the smooth muscle cells permits cells that are not directly activated by a relevant neuronal/hormonal signal to be rapidly, albeit indirectly, recruited into the contraction or relaxation response.

To summarize, the main implication of gap junctions to the genetic therapy of erectile dysfunction is that their presence would ensure that only a fraction of the corporal smooth muscle cells would need to be genetically modified in order to affect rather global changes in corporal smooth muscle tone. This is of crucial importance, as it would minimize the necessity for utilizing more aggressive genetic incorporation strategies (e.g., adenoviral or retroviral incorporation) which have a concomitantly greater number of side effects and concerns (e.g., insertional mutagenesis or immunological reactions).

Second, the proposed gene therapy is designed to take advantage of the fact that relative subtle alterations in the balance between contracting and relaxing stimuli can result in profound alterations in erectile physiology and function. The goal of gene therapy is therefore to restore a more normal balance between contracting and relaxing stimuli following expression of an exogenous gene(s) that codes for physiologically relevant proteins in corporal smooth muscle; in this case, the maxi-K channel. In light of the multifactorial nature of erectile dysfunction in man, there may in fact be many distinct genetic therapy strategies that will be effective in the restoration of erectile potency. For example, it is worth noting that qualitatively similar effects on intracavernous pressure were observed following the intracavernous injection of an inducible form of the nitric oxide synthase enzyme in a rat model. Thus, if expression of these or other extrachromosomal genes can be maintained in humans for a period of weeks to months (as the preliminary data herein indicates), it is conceivable that a patient could obtain "normal" erections in the absence of any other exogenous manipulation, during this time period. Clearly, this would be a major advance over all currently

available therapies.

Taken together, these data are consistent with the supposition that increased maxi-K channel activity, following a single intracorporal injection of naked *hSlo* DNA is the result of the presence of a greater number of maxi-K channels on some fraction of corporal smooth muscle cells. This, in turn, results in a greater hyperpolarization for any given level of neural stimulus, presumably altering intracellular calcium mobilization/homeostasis, and thus promoting greater corporal smooth muscle relaxation. In conclusion, it seems reasonable to assume that the relatively stable transfection of corporal smooth muscle cells with the human smooth muscle maxi-K channel cDNA represents an important and physiologically relevant strategy for the novel molecular manipulation of corporal smooth muscle tone in the treatment of organic erectile dysfunction.

What is claimed is:

1. A method of regulating bladder smooth muscle tone in a subject comprising the introduction and expression of a DNA sequence encoding a protein involved in the regulation of smooth muscle tone into a sufficient number of cells of the subject to regulate bladder smooth muscle tone in the subject.

2. The method of Claim 1 wherein the cell is a bladder smooth muscle cell.

3. The method of Claim 1 wherein the protein regulates relaxation.

4. The method of Claim 3 wherein the protein is selected from the group consisting of connexin, nitric oxide synthase, guanylate cyclase, adenylate cyclase, protein kinase G, protein kinase A, potassium channels, calcium channels, or any combination thereof.

5. The method of Claim 1 wherein the protein regulates contraction.

6. The method of Claim 5 wherein the protein is selected from the group consisting of connexin 43, alpha 1 receptor or the endothelin 1 receptor, phospholipase C, diacylglycerol, protein kinase C, myosin light chain kinase, calmodulin, potassium channels, calcium channels, or any combination thereof.

7. The method of Claim 1 wherein the protein encoded by the DNA inhibits a protein that regulates contraction of smooth muscle.

8. The method of Claim 7 wherein the protein is selected from the group consisting of connexin 43, alpha 1 receptor or the endothelin 1 receptor, phospholipase C, diacylglycerol, protein kinase C, phospholipase C, myosin light chain kinase, calmodulin, potassium channels, calcium channels, or any combination thereof.

9. The method of Claim 7 wherein the protein encoded by the DNA inhibits a protein that regulates relaxation of smooth muscle.

10. The method of Claim 9 wherein the protein is selected from the group consisting of connexin, nitric oxide synthase, guanylate cyclase, adenylate cyclase, maxi-K channel, protein kinase G, protein kinase A, potassium channels, calcium channels, or any combination thereof.

11. The method of Claim 9 wherein the protein is maxi-K channel or K(ATP).

12. The method of Claim 10 wherein the protein that regulates relaxation of smooth muscle is connexin 43.

13. The method of Claim 1, wherein the subject has bladder dysfunction.

14. The method of Claim 13, wherein said bladder dysfunction results from heightened contractility of the smooth muscle.

15. The method of Claim 13, wherein said bladder dysfunction results from impaired contraction of smooth muscle.

16. The method of Claim 13, wherein said bladder dysfunction results from interstitial cystitis.

17. The method of Claim 1 wherein the DNA sequence is introduced by a method selected from the group consisting of instillation therapy, electroporation, DEAE Dextran, cationic liposome fusion, protoplast fusion, by creation of an *in vivo* electrical field, DNA coated microprojectile bombardment, injection with recombinant replication-defective viruses, homologous recombination, and naked DNA transfer.

18. The method of Claim 1 wherein the DNA sequence is genomic DNA or cDNA.

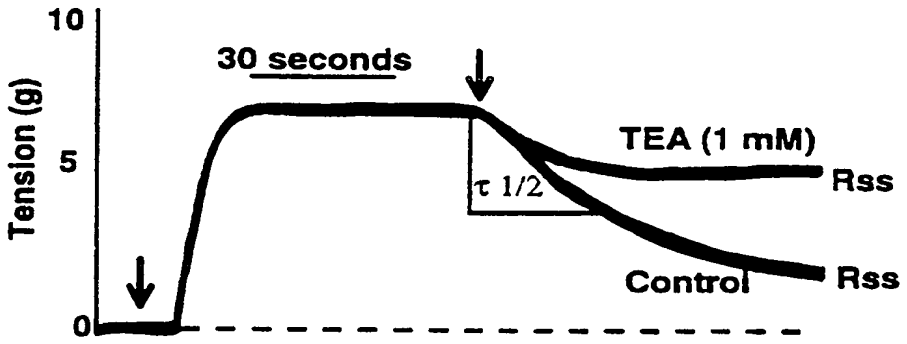

FIG. 1

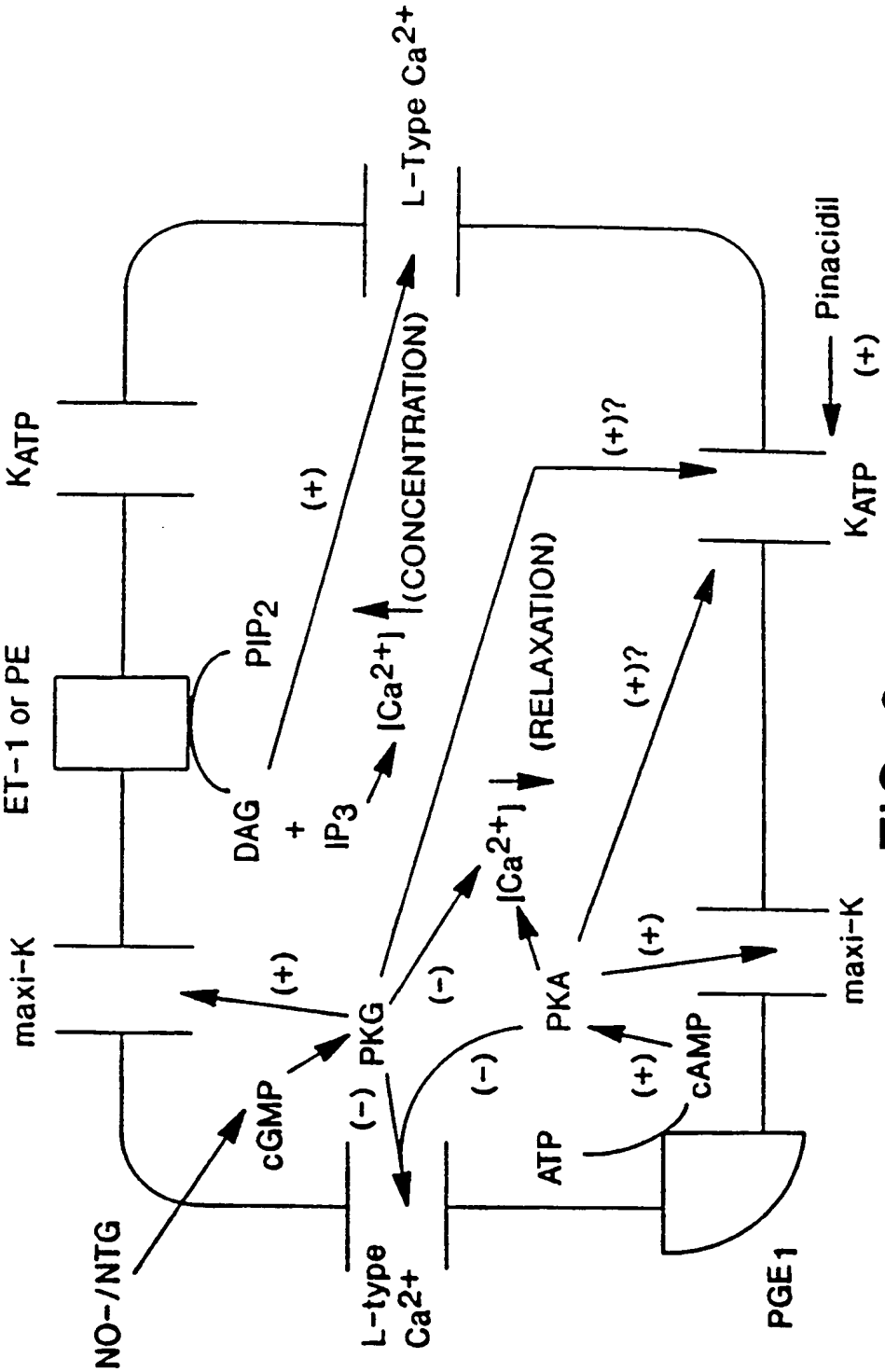

FIG. 2

Inside cell:
$[Ca^{2+}]$ = 100 nM
[K+] = 150 nM

Outside cell:
$[Ca^{2+}]$ = 2.0 mM
[K+] = 5 mM

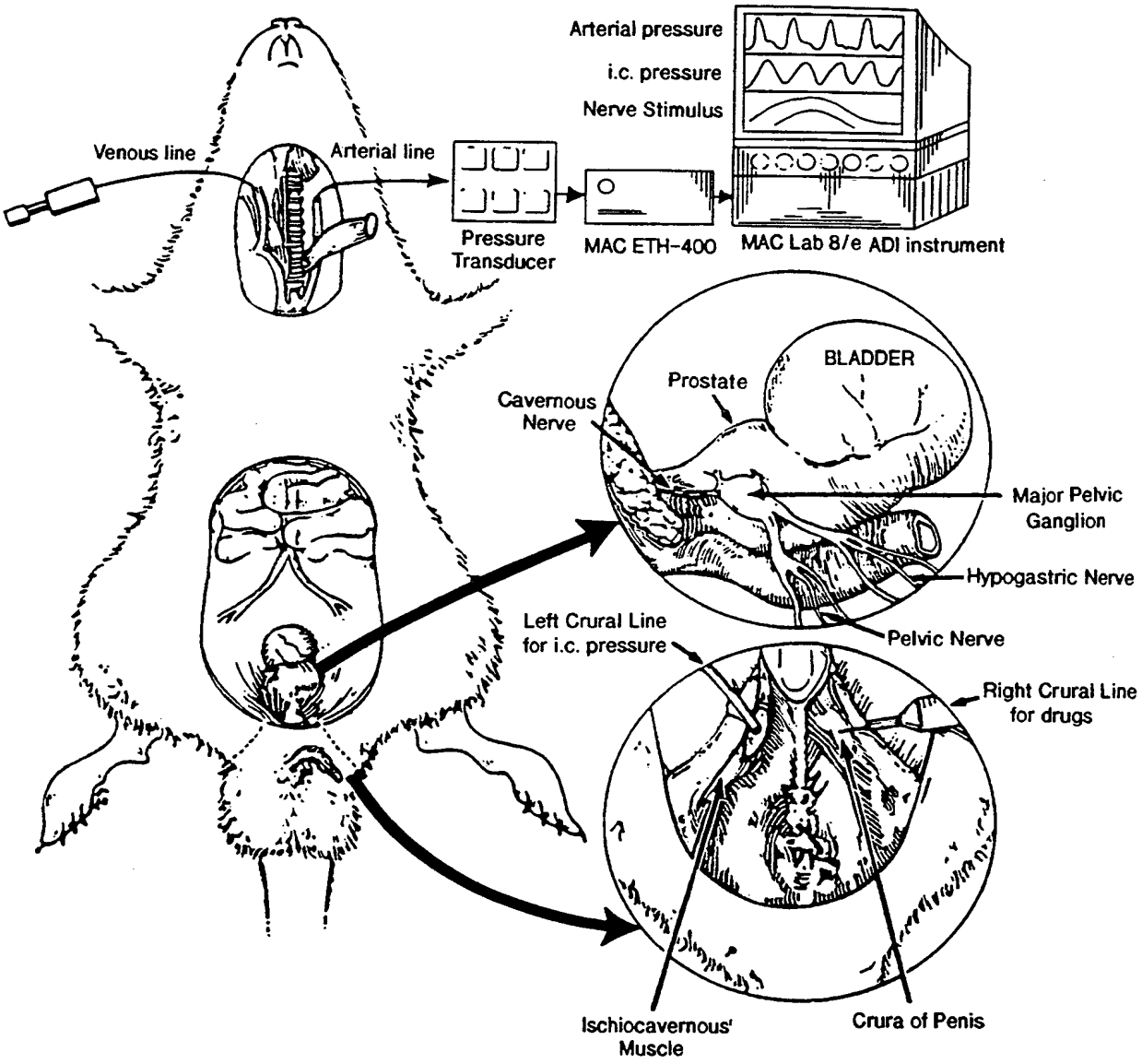
Arterial pressure
i.c. pressure
Nerve Stimulus
Venous line
Arterial line
Pressure Transducer
MAC ETH-400
MAC Lab 8/e ADI instrument
BLADDER
Prostate
Cavernous Nerve
Major Pelvic Ganglion
Hypogastric Nerve
Pelvic Nerve
Left Crural Line for i.c. pressure
Right Crural Line for drugs
Ischiocavernous Muscle
Crura of Penis

FIG. 3

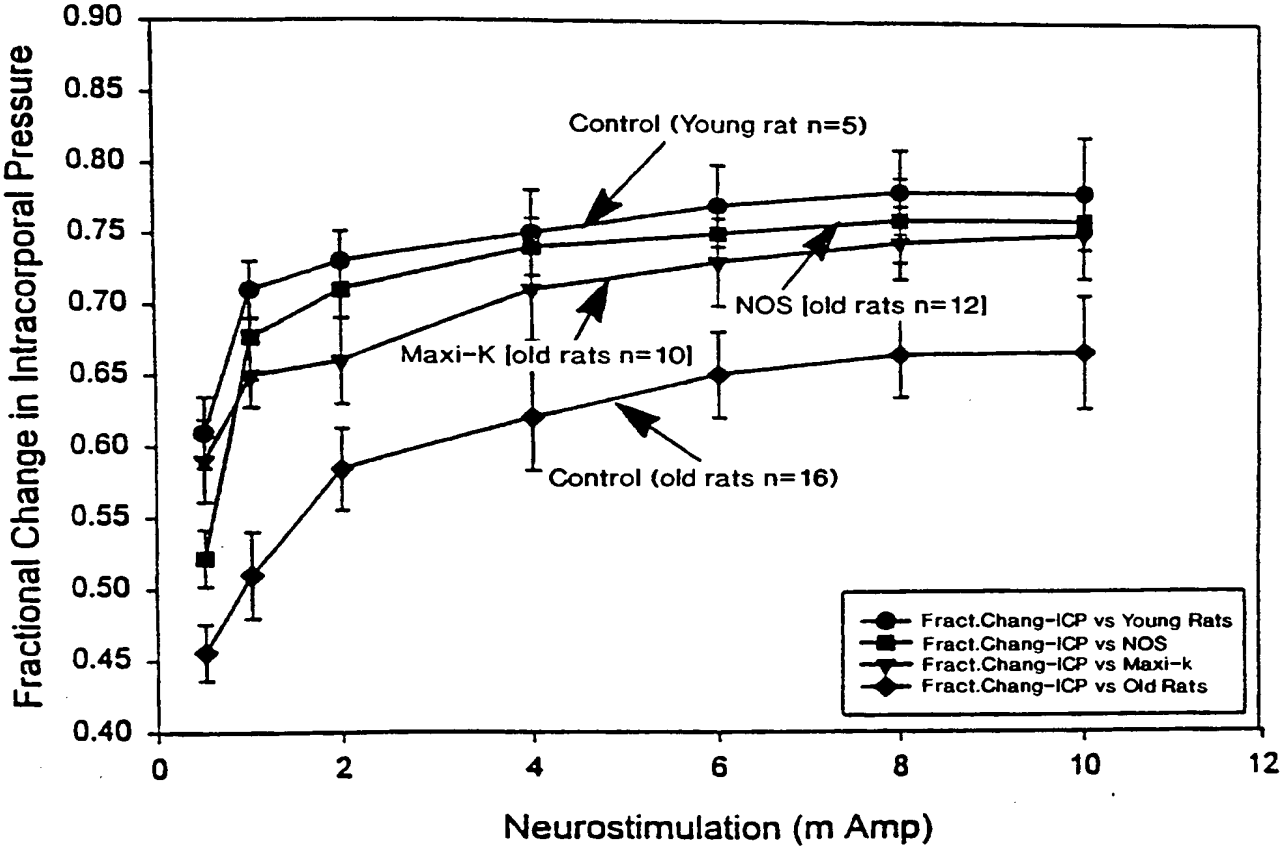
Fractional Change in Intracorporal Pressure
0.90
0.85
0.80
0.75
0.70
0.65
0.60
0.55
0.50
0.45
0.40
0
2
4
6
8
10
12
Neurostimulation (m Amp)
Control (Young rat n=5)
NOS [old rats n=12]
Maxi-K [old rats n=10]
Control (old rats n=16)
Fract.Chang-ICP vs Young Rats
Fract.Chang-ICP vs NOS
Fract.Chang-ICP vs Maxi-k
Fract.Chang-ICP vs Old Rats

FIG. 4

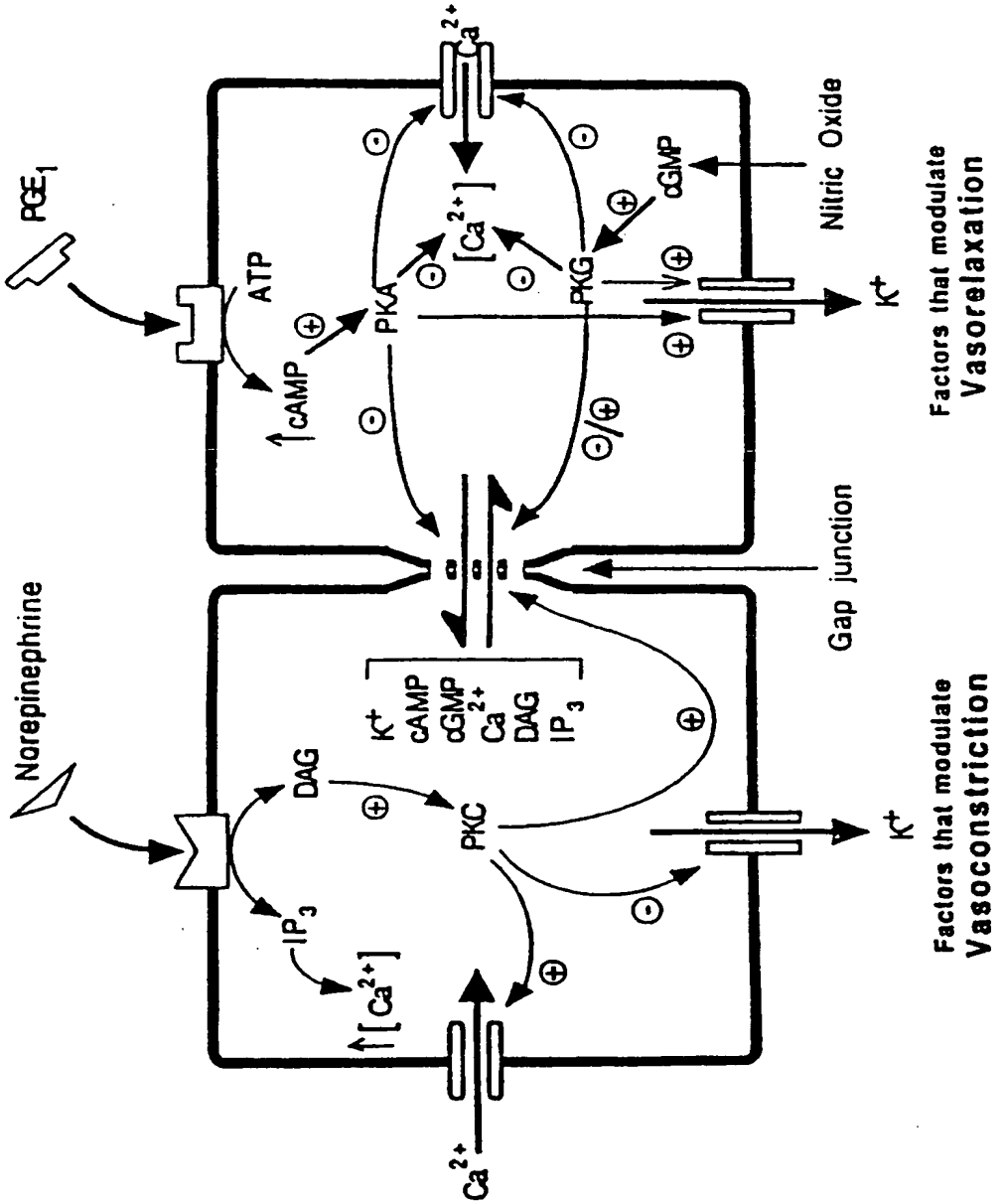
PGE1
ATP
cAMP
PKA
[Ca2+]
Ca2+
cGMP
Nitric Oxide
PKG
K+
Factors that modulate
Vasorelaxation
Gap junction
Norepinephrine
DAG
IP3
[Ca2+]
PKC
K+ cAMP cGMP Ca2+ DAG IP3
K+
Factors that modulate
Vasoconstriction
Ca2+

FIG. 5

FIG. 6

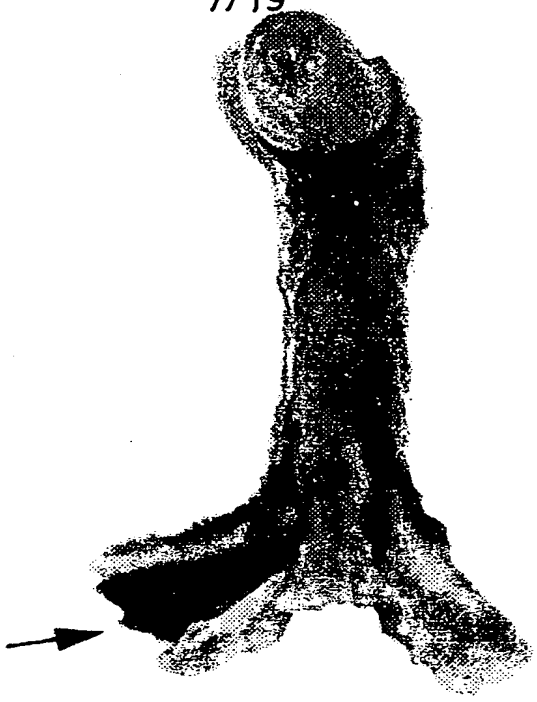

FIG. 7A

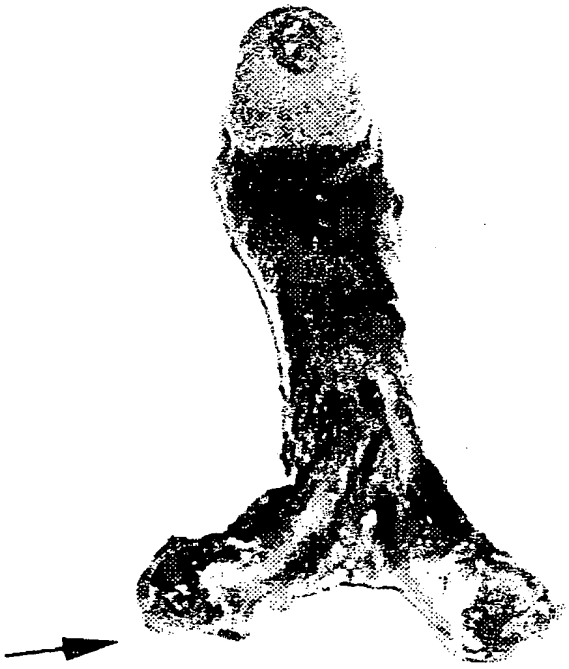

FIG. 7B

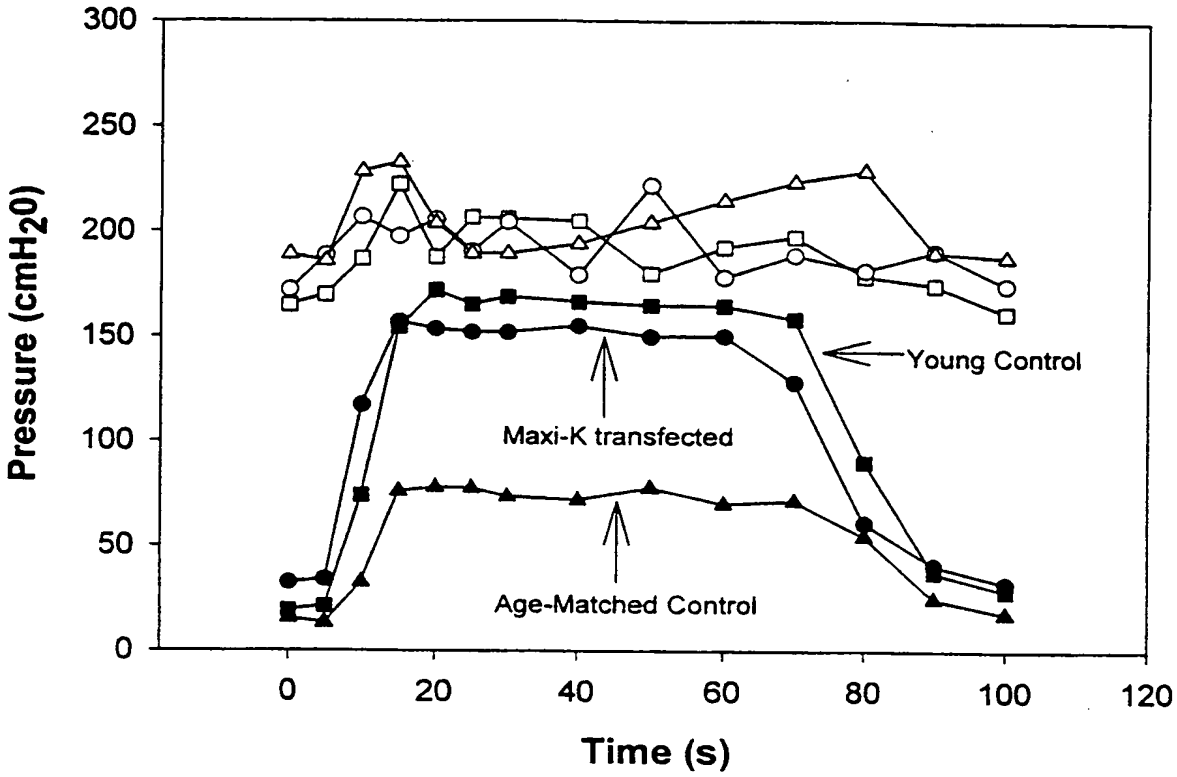
300
250
200
150
100
50
0
Pressure (cmH20)
0
20
40
60
80
100
120
Time (s)
Young Control
Maxi-K transfected
Age-Matched Control

FIG. 8

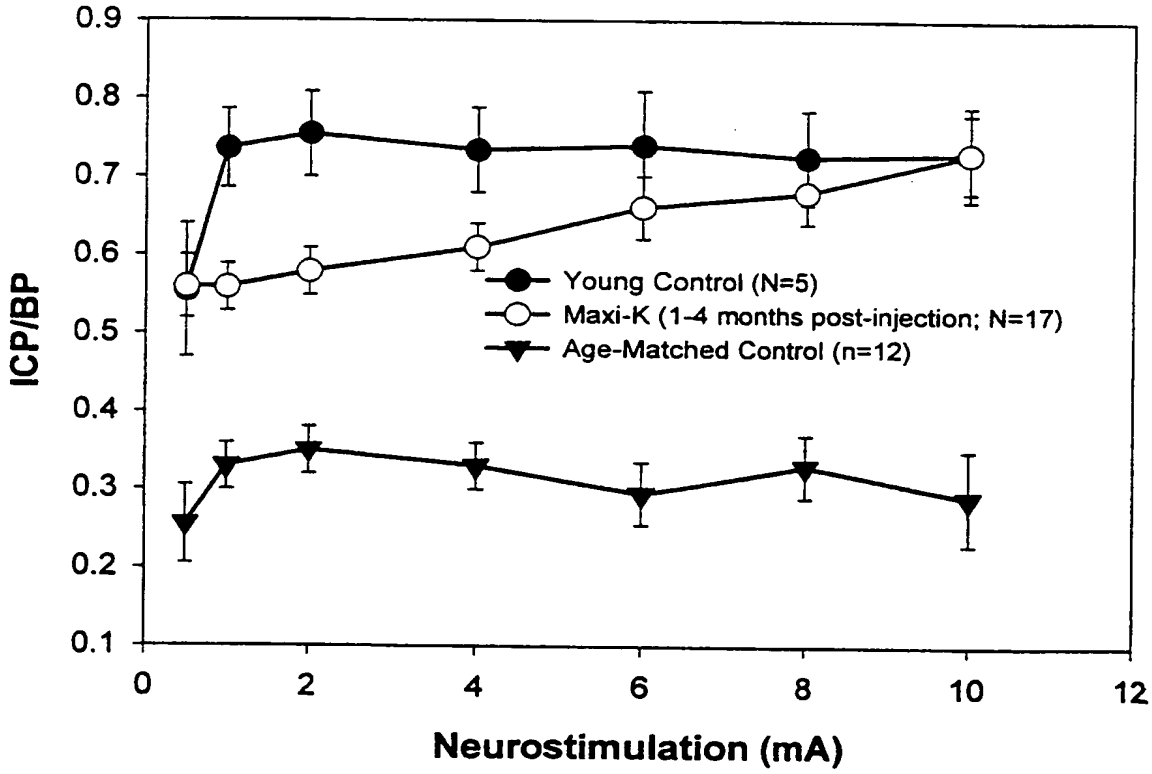
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
ICP/BP
0
2
4
6
8
10
12
Neurostimulation (mA)
Young Control (N=5)
Maxi-K (1-4 months post-injection; N=17)
Age-Matched Control (n=12)

FIG. 9

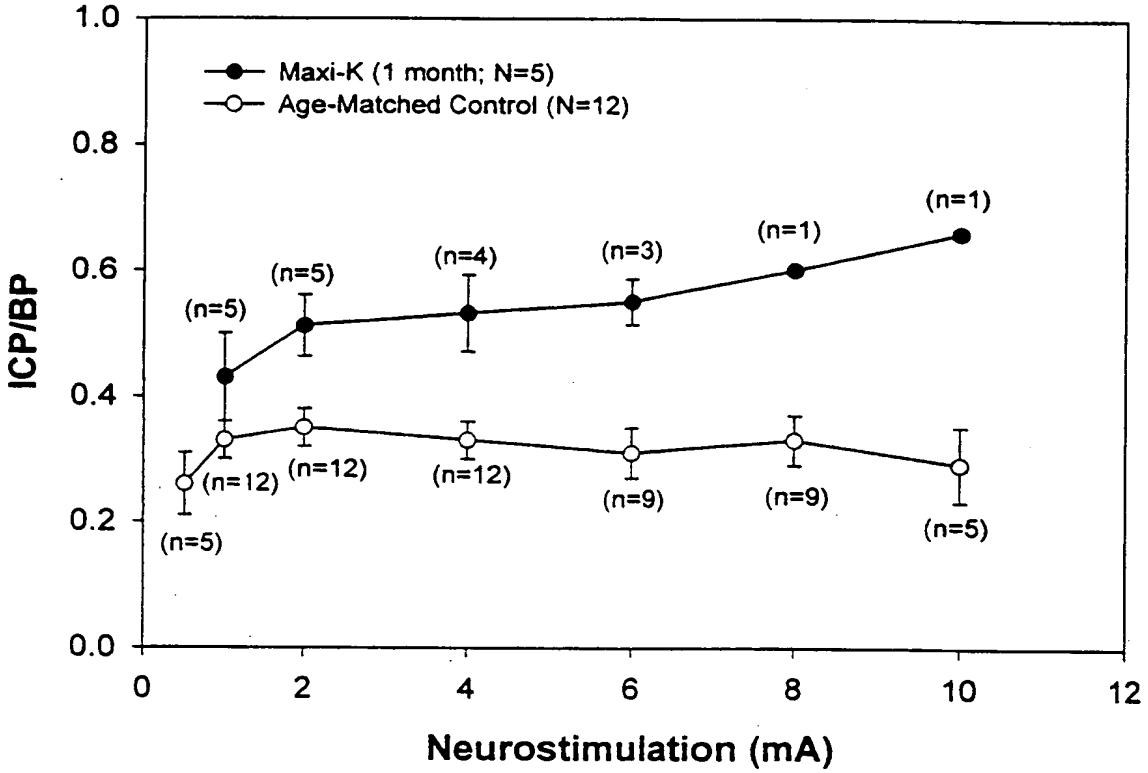

FIG. 10A

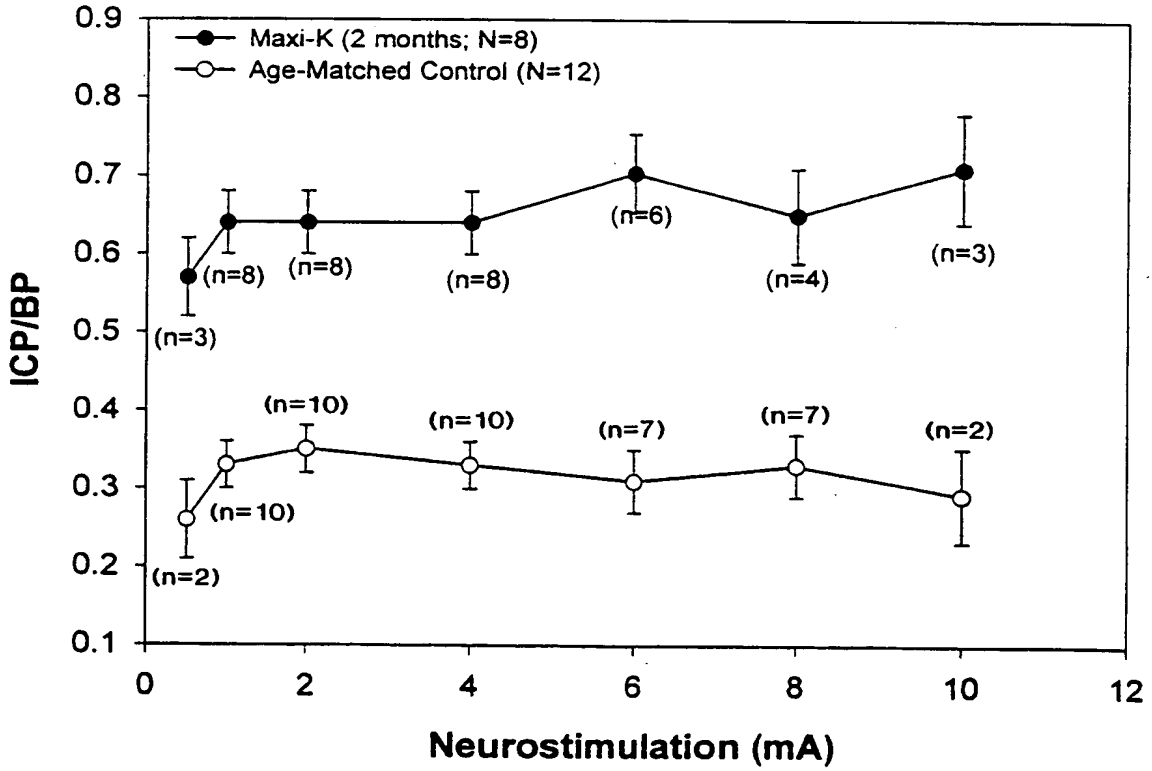

FIG. 10B

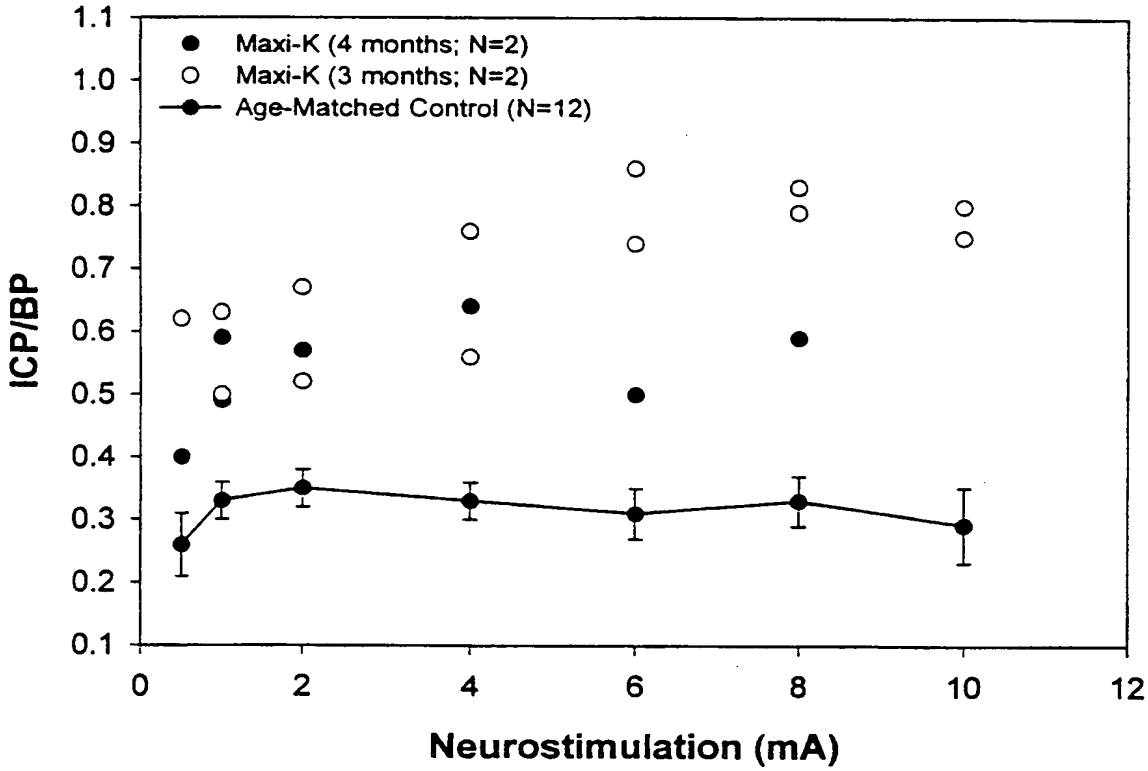
Maxi-K (4 months; N=2)
Maxi-K (3 months; N=2)
Age-Matched Control (N=12)
ICP/BP
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
2
4
6
8
10
12
Neurostimulation (mA)

FIG. 10C

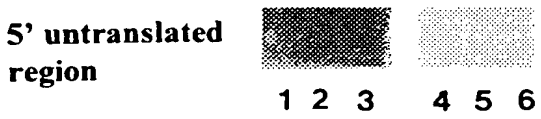

FIG. 11A

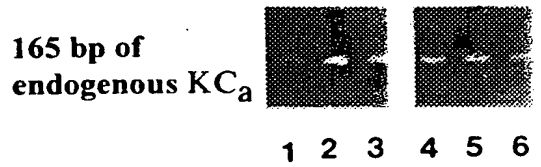

FIG. 11B

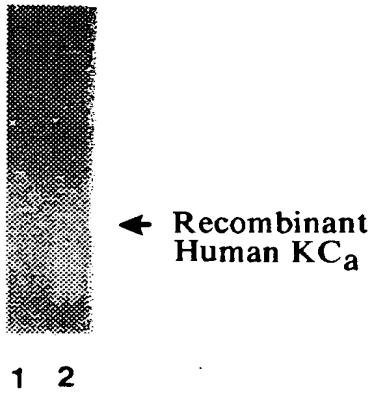

FIG. 11C

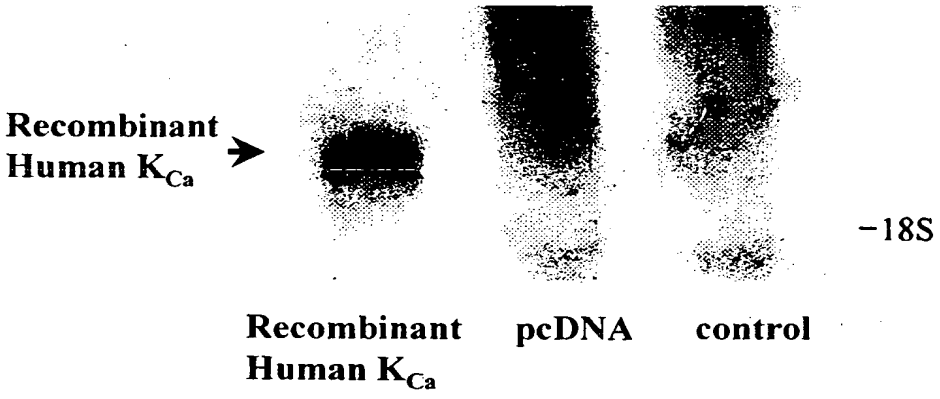
Recombinant
Human K_{Ca}
-18S
Recombinant
Human K_{Ca}
pcDNA
control

FIG. 12

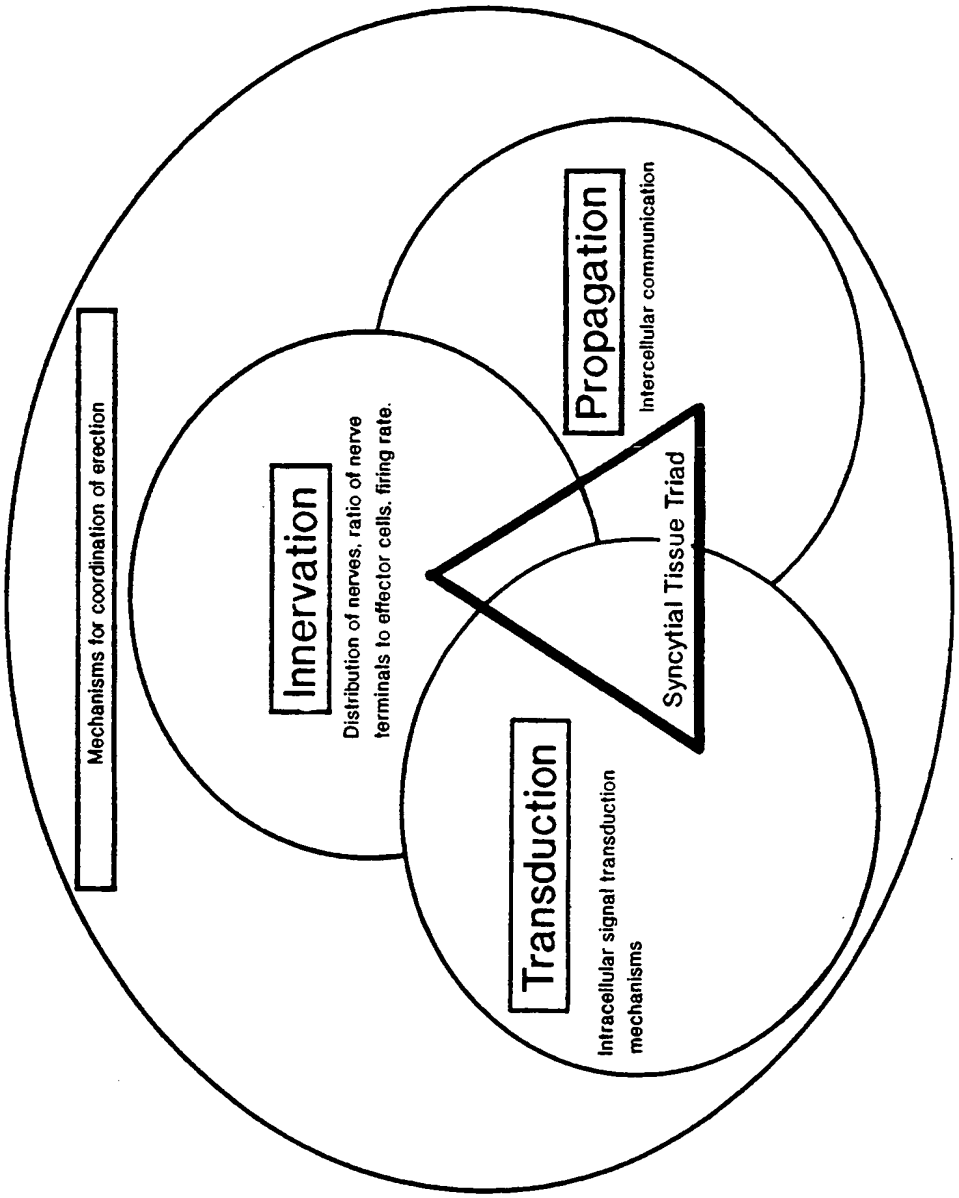
Mechanisms for coordination of erection
Innervation
Distribution of nerves, ratio of nerve terminals to effector cells, firing rate.
Propagation
Intercellular communication
Syncytial Tissue Triad
Transduction
Intracellular signal transduction mechanisms

FIG. 13

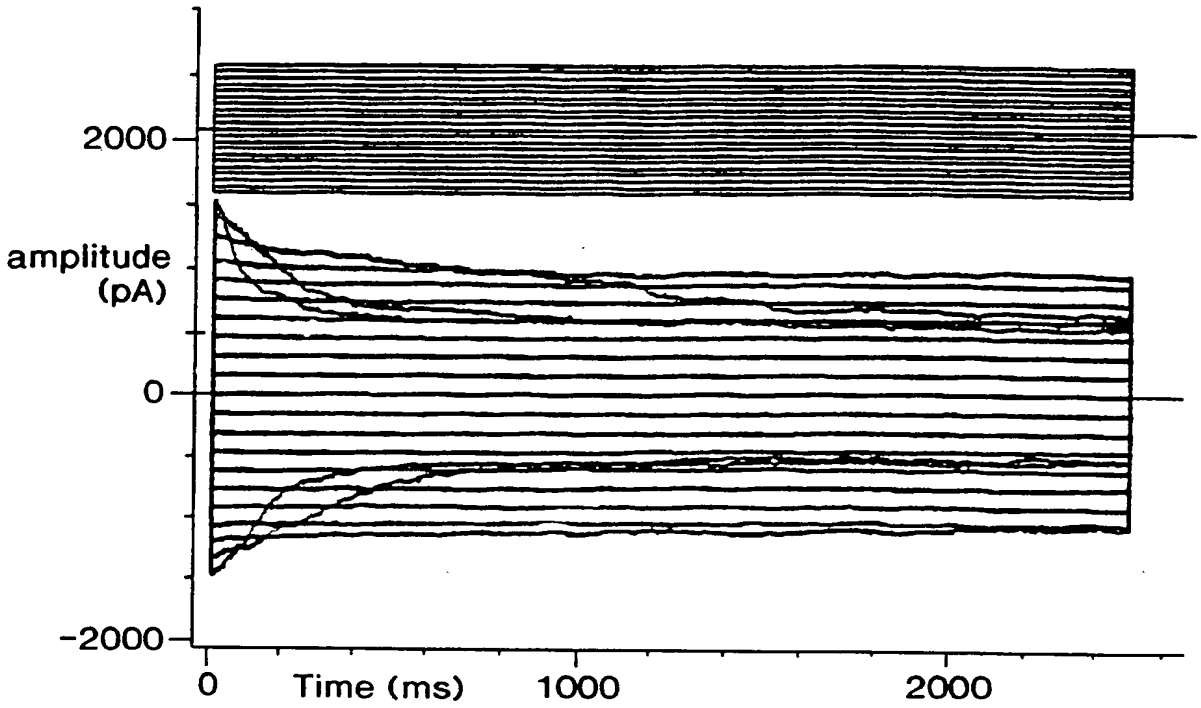

FIG. 14A

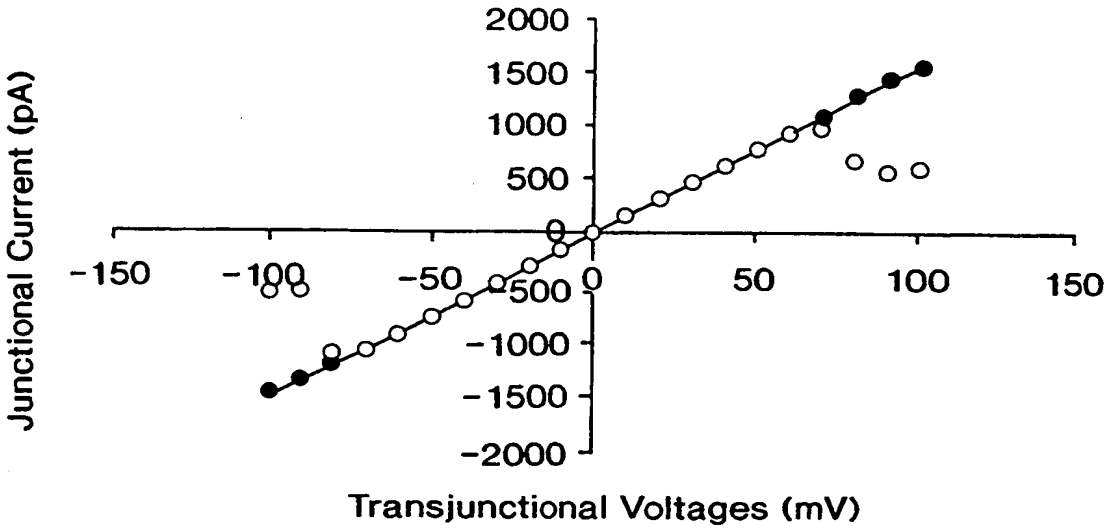

FIG. 14B

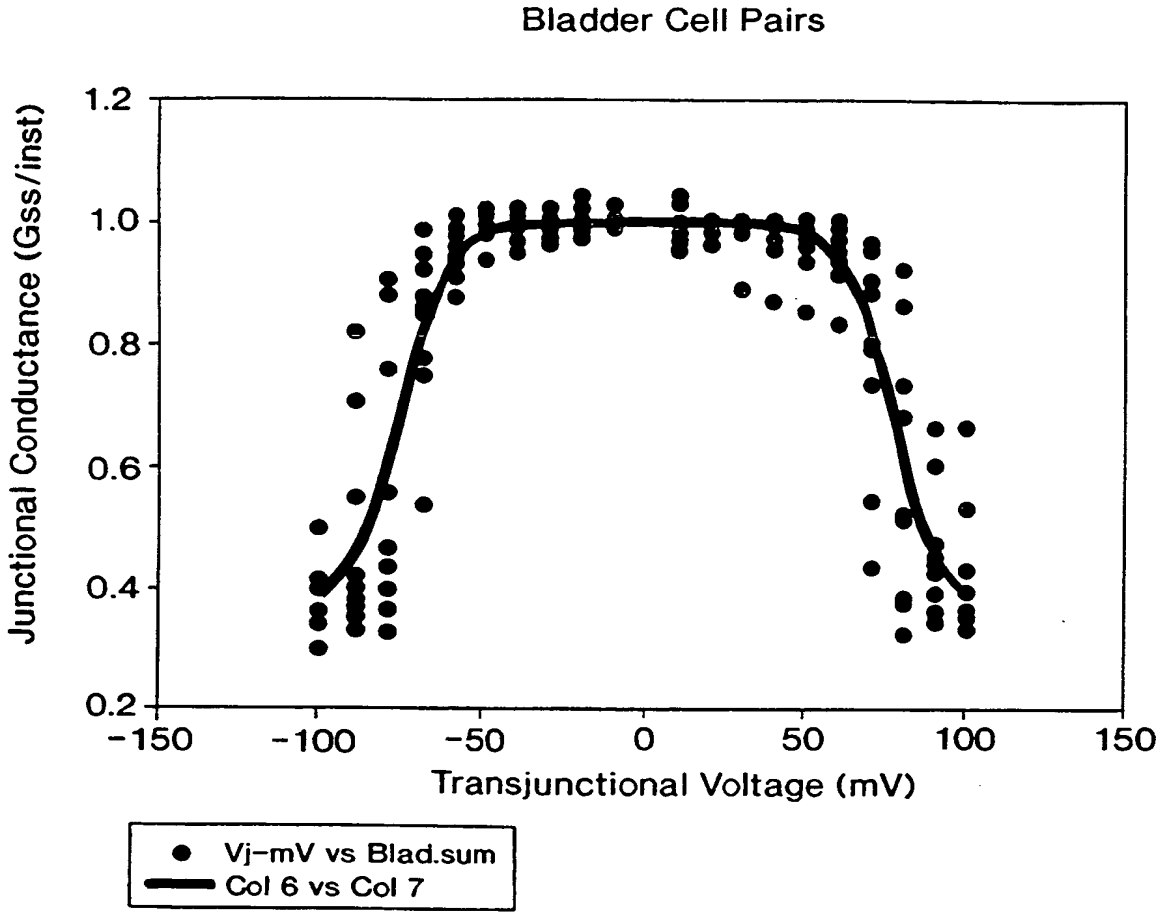
Bladder Cell Pairs
Junctional Conductance (Gss/inst)
1.2
1.0
0.8
0.6
0.4
0.2
-150
-100
-50
0
50
100
150
Transjunctional Voltage (mV)
Vj-mV vs Blad.sum
Col 6 vs Col 7

FIG. 15

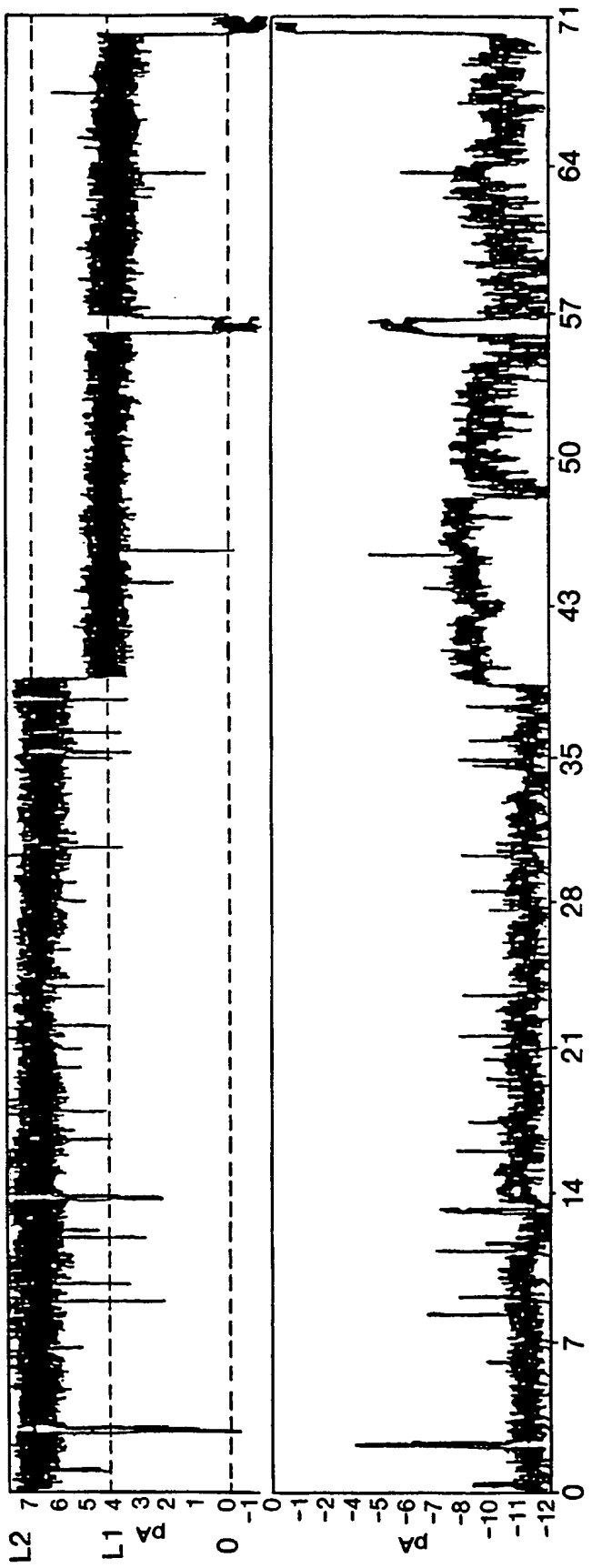

FIG. 16A

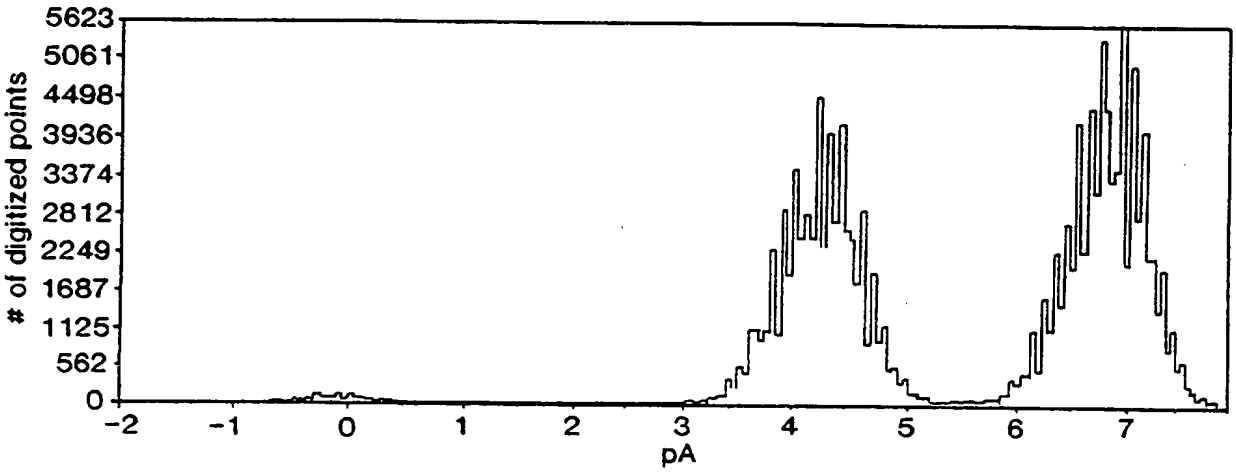

FIG. 16B

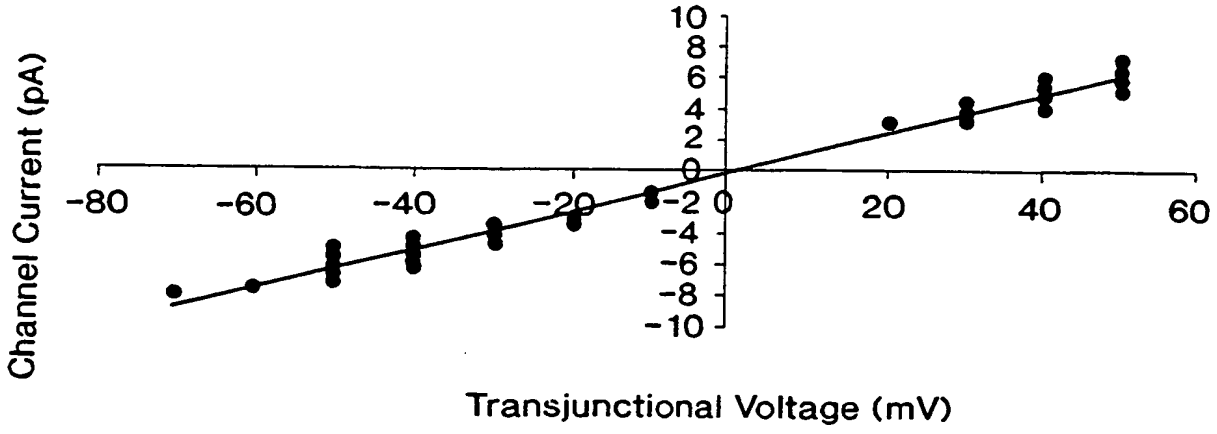

FIG. 16C

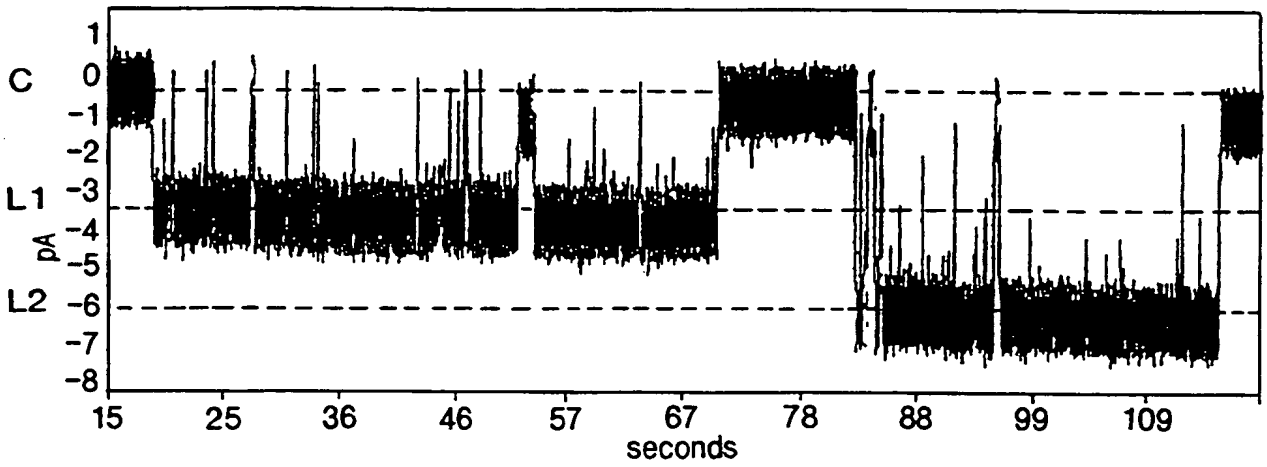

FIG. 17A

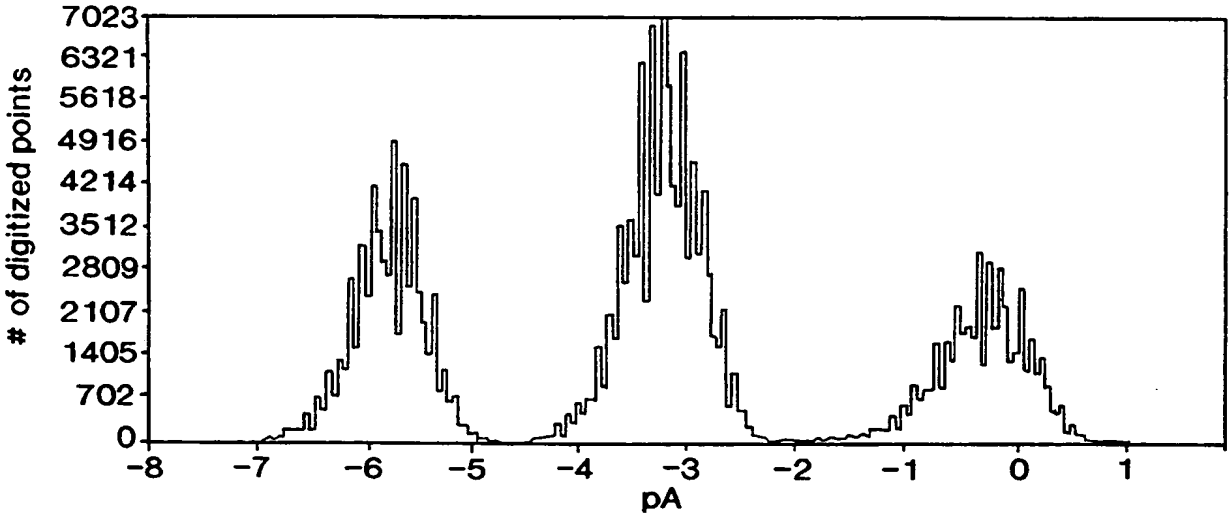

FIG. 17B

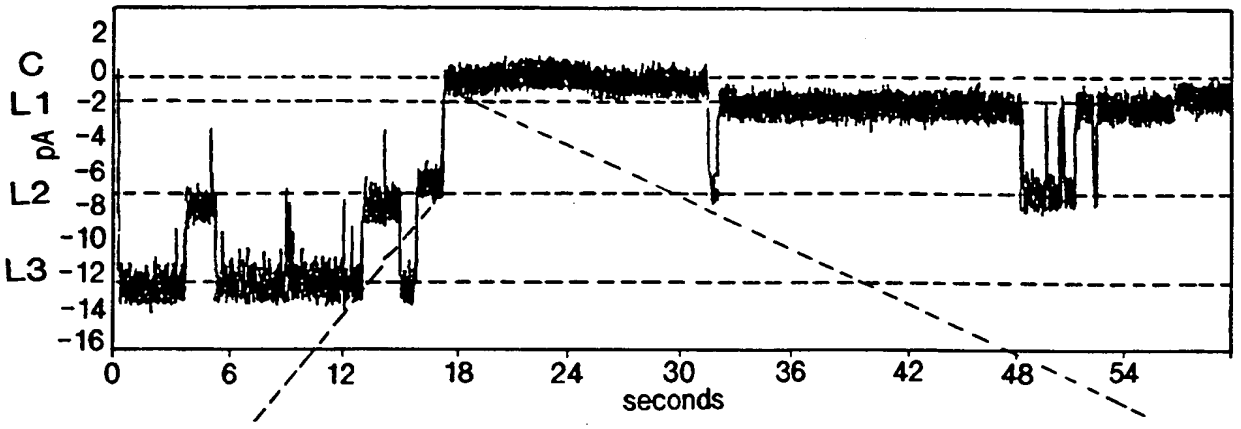

FIG. 18A

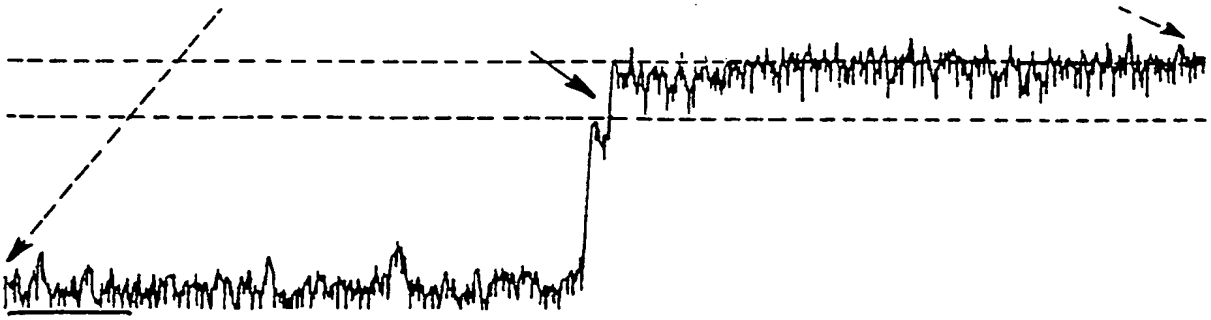

FIG. 18B

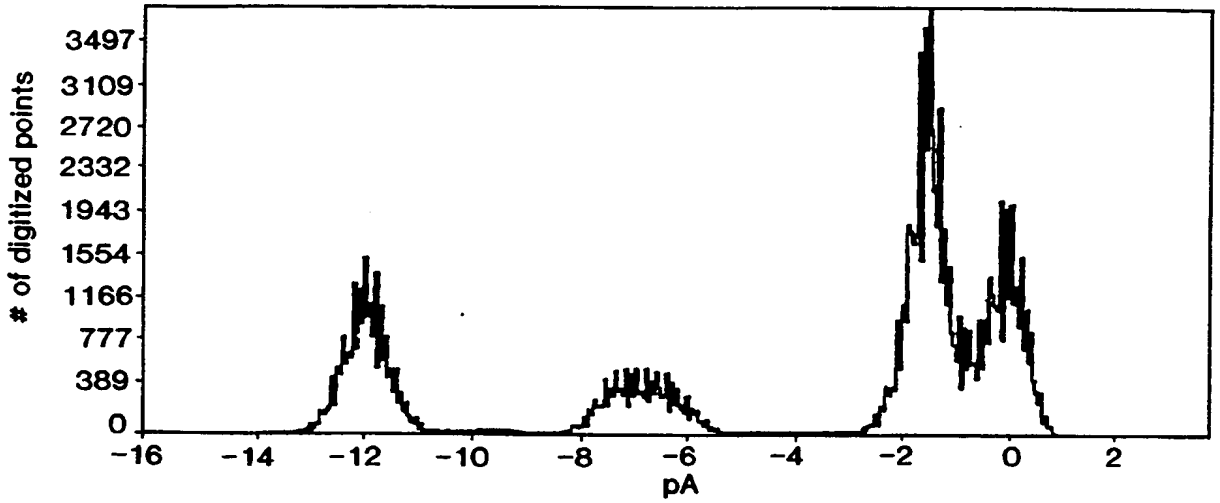

FIG. 18C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/18912

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :A61K 41/00; C12Q 1/68
US CL :514/44; 435/6

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 435/6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, EMBASE, SCISEARCH, CAPLUS, BIOSIS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	FAN et al. AN ANALYSIS OF THE MAXI-K^+ (K_{Ca}) CHANNEL IN CULTURED HUMAN CORPORAL SMOOTH MUSCLE CELLS. Journal of Urology. March 1995, Vol. 153, pages 818-825, see entire document.	1-18
Y	McCOBB et al. A human calcium-activated potassium channel gene expressed in vascular smooth muscle. Am. J. Physiol. September 1995, Vol. 269, No. 3, pages 767-777, see entire document.	1-18
Y	US 5,594,032 A (GONZALEZ-CADAVID et al.) 14 January 1997, see entire document.	1-18

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 30 NOVEMBER 1999	Date of mailing of the international search report 23 DEC 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer ANDREW WANG

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/18912

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CHRIST et al. Molecular Studies of Human Corporal Smooth Muscle: Implications for the Understanding, Diagnosing, and Treatment of Erectile Dysfunction. Molecular Urology. 1997, Vol. 1, No. 1, pages 45-54, see entire document.	1-18